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Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.jim.2014.10.009
Journal of Immunological Methods, 416, pp. 29-39, 2014-11-08

Characterization of high affinity anti-Internalin B VHH antibody fragments isolated from passively and artificially immunized repertoires.

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Abstract

The need for rapid and easy technologies for the detection of food-borne and environmental pathogens is essential for safe-guarding the health of populations. Furthermore, distribution of tainted food and water can have consequences which can affect whole economies. Antibodies and antibody fragments have been historically used in detection platforms due to their antigen specificity and robust physicochemical properties. In this study, we report the isolation and characterization of antibody fragments from the heavy chain antibody repertoire (VHH) of *Camelidae* which bind to the *Listeria monocytogenes* invasin, Internalin B (InlB), with specificity and high affinity. To the best of our knowledge, this is the first report of anti-InlB VHHs from camelids. These anti-InlB VHHs were not cross-reactive to the structurally-related *Listeria* invasin Internalin A (InlA). In addition, we demonstrate that antibody repertoires from passively immunized animals are good sources of specific binders to environmental pathogens, demonstrating that active immunization of animals may not be necessary in such cases. These antibody fragments are potential reagents to be used in the development of detection technologies.

Introduction

Listeria monocytogenes is a Gram-positive, food-borne bacterial pathogen which is the causative agent of listeriosis [1]. Although this bacterium is ubiquitously found in the environment, individuals with healthy immune systems are able to combat infection. However, the very young, the very old and pregnant women are high risk populations particularly sensitive to *L. monocytogenes*; infections can result in a mortality rate between 25-30% and, in pregnant women, spontaneous abortions [2].

Regular, thorough screening for the presence of food-borne bacterial pathogens is critical to safe-guard food sources which are to be widely distributed within a population. The importance of such requirements has been highlighted in the recent *Listeria monocytogenes* outbreaks which occurred in Canada [3] and the U.S. [4]. Although outbreaks are relatively rare, distribution of *L. monocytogenes* tainted food products have devastating health and economic consequences [5].

The current methodologies used to screen for the presence of *Listeria* spp. and in particular, *L. monocytogenes*, require specialized equipment and lengthy protocols [6]. Development of a rapid, sensitive and easy-to-use detection platform not reliant on expensive technologies, that could give a visual yes or no result, would be of great value to those tasked with routine screening procedures.

Many biosensor technology platforms rely on appropriate biorecognition elements for both efficiency and selectivity. Antibodies have been used as biorecognition reagents since the 1960s [7], and have recently been successfully used to detect pathogenic bacteria with great sensitivity [8].

In the Camelidae family, a subset of circulating gamma-immunoglobulin is heavy-chain only (HCAb) [9]. The variable heavy chain domain of a heavy chain antibody (VHH) retains antigen binding ability and exhibits high chemical, proteolytic and thermal stability [10-13], making it an ideal biorecognition reagent platform for biosensor production.

Here, we report the isolation and characterization of VHHs from a previously constructed naïve llama-alpaca-camel (LAC) phage library [14] and a phagemid library constructed from B cells isolated from an immunized llama which bind the *Listeria* invasin, Internalin B (InlB).

InlB is a member of the Internalin family of proteins, first discovered in *L. monocytogenes*. Internalins are characterized by the presence of a leucine-rich repeat (LRR) domain, and their function in pathogenesis [15]. Internalin A (InlA) and InlB have been heavily studied, revealing their role in early *L. monocytogenes* infection. InlA is responsible for initiating infection by binding to E-cadherin present on intestinal epithelial cells [16], followed by InlB binding to c-Met [17] to induce bacterial uptake by non-phagocytic cells [18-21]. Once the bacterium has established infection of the host, InlB facilitates the entry of *L. monocytogenes* across the blood-brain barrier [22], the maternofetal barrier [23,24] and into the liver [25], producing the trademark signs of listeriosis. The tandem function of InlA and InlB is observed at the genomic level, as they are on the same operon [26], under control of strong transcription factors [27-29]. *inlB* is exclusive to the *L. monocytogenes* genome [30], whereas *inlA* has been discovered in non-pathogenic Listeria [31]. Most importantly for antibody isolation, [32]InlA and InlB are structurally related, with InlA containing 15 LRRs [33] compared to 7 in InlB [34].

Although conventional antibodies which recognize InlB have been reported [35-39], to the best of our knowledge, this is the first report of isolation of VHHs shown to bind to InlB. In addition, we demonstrate that so-called naïve, i.e. passively immunized, antibody repertoires can be robust sources of binders against environmental, bacterial pathogens. These antibodies are reagents which can potentially be used in the development of rapid, highly sensitive, specific and cost-effective *Listeria monocytogenes* and other environmental pathogen detection technologies.

Materials and Methods

i) Internalin B recombinant fragment expression and purification

The cDNA encoding the InlB-LRR antigen was synthesized by DNA2.0 (Menlo Park, CA, USA) and subcloned into the pJ414Express vector. The insert containing vector was used to transform chemically competent E. cloni EXPRESS BL21(DE3)pLysS cells (Lucigen, Middleton, WI, USA). One litre of 2YT media containing 17 μg/mL of chloramphenicol and 100 μg/mL of carbenicillin was inoculated with 20 mL of overnight culture and incubated at 37 °C to an OD_{600nm} of 0.92. Protein expression was induced with 1 mM IPTG at 37 °C for 3.5 hours. Cells were pelleted at 6000 × g, 4 °C, 15 minutes using a JA-10 158 rotor in a J2-21M/E centrifuge (Beckman Coulter, Brea, CA, USA) and stored at -20 °C until processed. To extract the protein, cells were resuspended in 80 mL of 20 mM NaP*i* pH 7.4 buffer containing 10 mM imidazole, 500 mM NaCl (IMAC binding buffer) and 1 mL of 100 mM phenylmethanesulfonyl fluoride (PMSF). The resuspended cells were sonicated 6 X 30 seconds with 1 minute rests using a Sonifier 450 (Branson Sonic Power Company, Danbury, CT, USA) at an output control setting of 8 and a duty cycle setting of 80%. The lysate was centrifuged at 18000 × g, 4 °C, 30 minutes using a JA-10 rotor (Beckman Coulter, Brea, CA, USA). The supernatant was collected and filtered through a 0.45 μm filter.

A 5 mL His-Trap FF column (GE Healthcare, Baie d'Urfé, QC, Canada) was equilibrated with 20 mM NaP*i* pH 7.4 buffer containing 500 mM NaCl and 10 mM imidazole (Bioshop, Burlington, ON, Canada). The filtered supernatant was loaded onto the column attached to a P1 peristaltic pump (GE Healthcare, Baie d'Urfé, QC, Canada) and washed with 100 mL of the equilibration buffer. The column was attached to an ÄKTA FPLC (GE Healthcare, Baie d'Urfé, QC, Canada) and eluted with 100 mL of 20 mM NaP*i* pH 7.4 containing 500 mM NaCl and 250 mM imidazole (Bioshop, Burlington, ON, Canada) (IMAC elution buffer), collecting 5 mL fractions. Twenty microlitre aliquots from peak fractions were

resolved in a 12.5% SDS-PAGE gel to identify InlB-LRR containing fractions using Coomassie staining. These fractions were pooled and desalted into 1x PBS pH 7.4 using a HiPrep 26/10 desalting column (GE Healthcare, Baie d'Urfé, QC, Canada). The protein was further purified using a Superdex 75 16/60 column (GE Healthcare, Baie d'Urfé, QC, Canada) into 1x PBS pH 7.4, collecting 5 mL fractions. Protein purity was assessed using Coomassie staining and highly pure fractions were pooled and concentrated to 4.5 mL using a 3 kDa MWCO spin filter (EMD Millipore, Mississauga, ON, Canada). The final concentration of the protein prep was determined using an ND-1000 Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) under denaturing conditions to be approximately 8.5 mg/mL.

ii) Internalin A recombinant fragment expression and purification

The cDNA encoding the InlA-LRR antigen was synthesized by DNA2.0 (Menlo Park, CA, USA) and subcloned into the pET26b vector. The insert containing vector was used to transform chemically competent E. cloni EXPRESS BL21 (DE3) cells (Lucigen, Middleton, WI, USA). One litre of LB media containing 1% (w/v) glucose and 50 μg/mL kanamycin was inoculated with 1 mL overnight culture. Cells were grown at 37 °C, 250 rpm to an OD_{600nm} of 0.5. To induce protein expression, IPTG solution was added to the culture at a final concentration of 1 mM and the culture was incubated for 4 hours at 37 °C, 250 rpm. Cells were pelleted at 4400 x g 4 °C, 15 minutes using a JA-10 158 rotor in a J2-21M/E centrifuge (Beckman Coulter, Brea, CA, USA) and stored at -20 °C until processed. Approximately 1 g of cell pellet was collected and stored. For protein extraction, the pellet was thawed and resuspended in 30 mL of IMAC binding buffer and 1 mL of 100 mM PMSF. The resuspended cells were incubated with 15 mg of lysosyme (USB, Cleveland, OH, USA) (3 mL of a 5 mg/mL stock) at room temperature until the solution became viscous (~ 20-30 mintues). The lysate was then incubated with 100 U of Benzonase nuclease (EMD Millipore, Mississauga, ON, Canada) to digest nucleic acid and "thin" the

lysate. The lysate was centrifuged at 17000 x g 4 °C, 30 minutes using a JA-10 rotor (Beckman Coulter, Brea, CA, USA). The supernatant was collected and filtered through a 0.45 μm filter.

The protein was purified from the lysate, assessed and concentrated as described in Section i. The final concentration of the protein prep was determined to be approximately 14.5 mg/mL using an ND-1000 Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

iii) Secondary structure evaluation of antigens using Far-UV CD spectroscopy

Both InlA-LRR and InlB-LRR purified products were buffer exchanged into 20 mM NaP*i* pH 7 containing 1 mM dithiothreitol [34] using an Amicon spin filter (EMD Millipore, Mississauga, ON, Canada). Fifty microlitres of antigen in 1x PBS and 10% glycerol were diluted into a total volume of 500 μL and centrifuged until the volume was brought back to 50 μL. This step was repeated four more times. Far-UV CD spectra were established at room temperature using a Jasco J-815 CD spectrometer (Easton, MD, USA). Briefly, the InlA and InlB proteins were buffer exchanged into 10 mM NaP*i* pH 7. A dilution of 0.1 mg/mL of InlA and 1.33 mg/mL of InlB were prepared and subjected to Far-UV scan at 20 °C. To demonstrate that secondary structure was being disrupted, the samples were heated to 95 °C and incubated for 5 minutes and the scan run again. Overlay of the 20 °C scan with that of the scan at 95 °C was carried out to demonstrate the loss of structure of each protein.

iv) Construction of immune library

An immune phagemid VHH display library was constructed using the pMED1 vector backbone[40], and lymphocytes derived from a male llama immunized with the InlB-LRR antigen. The immunization protocol was carried out by

Cedarlane Labs according to in-house protocols (Burlington, ON, Canada). Gerbu adjuvant (Gerbu Biotechnik GmbH, Heidelberg, Germany) was used for all injections except the first, for which Complete Freund's Adjuvant was used. Briefly, mononuclear blood cells were isolated using Lymphoprep™ (Axis-Shield, Dundee, UK) and RNA was extracted from 5-10 x 10^6 cells using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA). cDNA synthesis was carried out using First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) using the pd(N)6 primer provided in the kit and following the manufacturer's protocol. The following primers were used to amplify VHH genes from the cDNA pool [41]:

S = C/G; M = A/C; K = G/T

MJ1 Back	5'-GCCCAGCCGGCCATGGCCSMKGTGCAGCTGGTGGAKTCTGGGGGA-3'
MJ2 Back	5'-GCCCAGCCGGCCATGGCCCAGGTAAAGCTGGAGGAGTCTGGGGGA-3'
MJ3 Back	5'-GCCCAGCCGGCCATGGCCCAGGCTCAGGTACAGCTGGTGGAGTCT-3'
C_H2	5'-CGCCATCAAGGTACCAGTTGA-3'
C_H2b3	5'-GGGGTACCTGTCATCCACGGACCAGCTGA-3'
MJ7	5'-CATGTGTAGACTCGCGGCCCAGCCGGCCATGGCC-3'
MJ8	5'-CATGTGTAGATTCCTGGCCGGCCTGGCCTGAGGAGACGGTGACCTGG-3'

PCR products were digested with *SfiI* enzyme (New England Biolabs, Ipswich, MA, USA) and purified using a PCR purification kit (Qiagen, Mississauga, ON, Canada). Digested products were ligated into linearized pMED1 vector using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and purified using a PCR purification kit. Ligation products were used to transform electrocompetent *E. coli* TG1 cells (Lucigen, Middleton, WI, USA) to establish the immune phagemid library.

v) Panning using the naïve library and the immune library

The naïve LAC phage library built on a modified Fd genome [42] was used to isolate InlB-LRR binders. Approximately 10^{12} virions in total were used as input in the first of four rounds of panning. The following procedure was repeated for each round, using decreasing amounts of InlB-LRR antigen and increasing wash stringency every round. A single polystyrene well (Becton Dickinson, Mississauga, ON, Canada) was coated with InlB-LRR diluted in 1x PBS (pH 7.4, 100 µL). Another polystyrene well was coated with 350 µL of round-specific blocking buffer. One-percent (w/v) casein-PBS (pH 7.4) was used for rounds (I) and (III), while StartingBlock™T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA, USA) was used for rounds (II) and (IV), respectively. Both the InlB-LRR-containing well and the blocking well were sealed and incubated overnight at 4°C. The following morning, both wells were rinsed with 350 µL 1x PBS pH 7.4 and incubated at room temperature with blocking buffer in the InlB-LRR well and phage library in the blocked well. Incubation of the phage library with blocking buffer before exposure to InlB-LRR was carried out to eliminate any potential non-specific binders from the phage pool. After 1 hr at room temperature, the InlB-LRR-containing well was washed with 1x PBS (pH 7.4, 350 µL) and the phage library transferred from the blocked well to the InlB-LRR-containing well. The incubation time of phage library with the InlB-LRR-containing well was 2 hrs for round (I), 1.5 hrs for round (II) and 1 hr for rounds (III) and (IV), all performed at room temperature. Unbound, or loosely bound phage particles were then washed out of the InlB-LRR-containing well with 1x PBS (pH 7.4) containing 0.1% Tween20 (0.1%-PBST), and the remaining phage eluted with triethylamine (TEA) (100 mM, 100 µL) for 10 min, and neutralized with Tris-HCl (pH 7.4, 1 M, 50 µL). Eluted phage particles were used to infect 3 mL of log-phase *E. coli* TG1 cells (Agilent, Mississauga, ON, Canada) for 15 min at 37 °C with no shaking. Eluted phage were enumerated by plating 100 µL, 10 µL and 1 µL aliquots of phage-infected cells on separate 2YT/Tet plates and incubating overnight at 32 °C. The remaining cells were amplified by plating on 2YT/Tet for overnight incubation at 32 °C. The following morning, cells were scraped off of the amplification plates and cultured in 2YT/Tet at 28 °C with

vigorous shaking for 5 hrs. The cells were pelleted (10 min, 4°C, 3600 × g) and the supernatant cleared through a 0.22 µm syringe-driven filter. PEG-NaCl (20% v/v PEG, 2.5 M NaCl) and supernatant were mixed at a 1:5 ratio, phage particles allowed to precipitate for 1 hr on ice, pelleted (30 min, 4 °C, 3600 × g) and resuspended in 1x PBS (pH 7.4) [43]. A serial dilution of purified phage particles, ranging from 10^{-4} to 10^{-14} was made using 1x PBS pH 7.4 as a diluent and used to infect log-phase TG1 cells. These phage-infected cells were plated on 2YT/Tet and incubated overnight at 32 °C to enumerate amplified phage.

Panning using the immune phagemid library was carried out as described above except that eluted phagemid particles were used to infect log phase TG1 cells and then superinfected with M13KO7 helper phage (New England Biolabs, Ipswich, MA, USA) to rescue the phagemid particles. The particles were concentrated via precipitation using the PEG-NaCl protocol described above.

vi) Phage and phagemid ELISA

Colonies from phage enumeration plates were picked and seeded in 250 µL 2YT/Tet per well in round-bottom tissue culture plates (Becton Dickinson, Mississauga, ON). Cultures were grown overnight at 30 °C with gentle shaking. A Maxisorp™ microtiter plate (Thermo Fisher Scientific, Waltham, MA, USA) was coated with 10 µg InlB-LRR per well, using 1x PBS pH 7.4 as diluent. The next morning, the plate was rinsed with PBS (pH 7.4, 350 µL) and blocked with 350 µL StartingBlock™T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 hr. Blocking buffer was removed, and the plate rinsed with 1x PBS pH 7.4. The tissue culture plate was centrifuged at 2000 × g for 5 min at 4 °C in a Sorvall Legend RT+ table top centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) to pellet TG1 cells and 100 µL of phage-containing supernatant was aspirated without disturbance of the cell pellet, and added to the antigen-coated microtiter plate. Phage particles were allowed to bind for 1 hr at room temperature. Appropriate controls were also included in the plate. Plates were washed 5 times with 0.1%-PBST and

incubated with 100 µL anti-M13 antibody conjugated to horseradish peroxidase (HRP) (GE Healthcare, Baie d'Urfé, QC, Canada) (used at 1:5000 diluted in 1x PBS pH 7.4) for 1 hr at room temperature. Plates were washed 5 times with 0.1%-PBST and incubated with 100 µL 3,3',5,5' – tetramethylbenzidine (TMB) peroxidase substrate (KPL, Gaithersburg, MD, USA). Color development was stopped with phosphoric acid (1 M, 100 µL), and the microtiter plate read at 450 nm on a MultiSkan FC spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Four negative control wells received primary incubation with conditioned growth media (2YT/Tet incubated overnight alongside cultured TG1 clones) in place of phage-containing culture supernatant. They were coated and developed in an identical manner to sample wells.

vii) VHH expression and purification

VHH genes were subcloned into the pSJF2H vector as previously described [44]. Positive clones were used to inoculate a 100 mL culture of LB/Carb and grown overnight at 37 °C with vigorous shaking. The entire overnight culture was used to seed 900 mL of LB/Carb, and the resulting 1 L culture was grown at 32 °C with vigorous shaking. At OD_{600} = 0.9, cultures were induced with IPTG at a final concentration of 0.5 mM and grown overnight at 32 °C. The next morning, cell solutions were centrifuged at 6000 × g, 15 min, 4 °C and pellets resuspended in 30 mL of cold TES buffer (0.2 M Tris-HCl (pH 8.0), 0.5 M sucrose, 0.5 mM EDTA) supplemented with 1 mM PMSF and incubated on ice for 30 min, after which 30 mL of cold 1/8-TES buffer (diluted in dH_20) was added and the cells incubated for another 30 min. Cell solutions were centrifuged and the supernatant harvested and dialyzed against Buffer A (20 mM phosphate buffer (pH 7.4), 0.5 M NaCl, 10 mM imidazole) overnight at 4 °C, followed by filtration through a 0.45 µm membrane (EMD Millipore, Mississauga, ON, Canada). Dialyzed supernatant was loaded onto nickel-charged 5 mL HisTrap™ FF columns (GE Healthcare, Baie d'Urfé, QC, Canada). The VHHs were eluted using gradient elution (buffer containing 500 mM imidazole). Eluted fractions

containing VHHs were pooled and the buffer exchanged for 1x PBS pH 7.4 using a HiPrep™ 26/10 desalting column (GE Healthcare, Baie d'Urfé, QC, Canada). VHHs were further purified using a Superdex™ 75 16/60 size exclusion column (GE Healthcare, Baie d'Urfé, QC, Canada). All chromatography was performed using an ÄKTA FPLC system (GE Healthcare, Baie d'Urfé, QC, Canada). Size exclusion fractions containing VHHs were analyzed by SDS-PAGE for content, and pure fractions pooled and stored at 4 °C until use. Sodium azide was added at 0.025% (w/v) as a microbicidal agent.

To distinguish VHHs isolated from the immunized library from those isolated from the LAC library, the former are designated with the "Imm" prefix (ex., Imm-Rn-m vs. Rn-m). For all VHHs, R designates "Round", "n" designates the stage of panning, and m is the clone number isolated from that "n" round.

viii) Binding kinetics analysis of VHHs to recombinant antigen using SPR

Prior to kinetic analysis, purified InlB-LRR VHHs were sized using a size exclusion column to collect homogeneous peaks containing monomeric protein after reconstitution in 1x PBS (pH 7.4). VHHs were injected over a Superdex™ 75 HR 10/30 size exclusion column (GE Healthcare, Baie d'Urfé, QC, Canada) and buffer exchanged to Biacore™ running buffer, HBS-EP (pH 7.4, 10mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) P20). Chromatography was performed using an ÄKTA FPLC system (GE Healthcare, Baie d'Urfé, QC, Canada).

The Biacore 3000 system (GE Healthcare, Baie d'Urfé, QC, Canada) was used for determination of kinetics of VHH binding to InlB-LRR. The carboxyl groups forming the bioconjugation layer on a CM5 chip (GE Healthcare, Baie d'Urfé, QC, Canada) were activated using 0.4 M 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M *N*-Hydroxysuccinimide (NHS) diluted in dH_20. InlB-LRR was diluted in sodium acetate buffer (pH 4.0, 10 mM) and injected over the activated carboxyl groups. Unreacted carboxymethyl groups were

deactivated with an injection of ethanolamine (pH 8.5, 1 M). All reagents used to produce the InlB-LRR-conjugated sensor chip were injected at 5 µL/min for 7 min. The InlB-LRR surface was stable and produced repeatable data when regenerated using 40 sec glycine (pH 2.5, 10 mM) pulses at a flow rate of 20 µL/min.

Purified InlB-LRR binders were diluted in HBS-EP to various concentrations, and 200 µL of each concentration injected over the InlB-LRR surface at a rate of 20 µL/min. All analyses were performed at 25 °C with surface regeneration after each concentration tested. All data was analyzed with BIAevaluation™ 4.1 software.

ix) Recognition of biological antigen

The wild-type *L. monocytogenes* strain EGD serovar 1/2a (EGD) and its derived *inlA* and *inlB* knockout cell lines (ΔinlA and ΔinlB, respectively) [25] were cultured at 37 °C overnight in 2YT with vigorous shaking. Two 15 mL cultures were prepared for each strain. The following day, InlB was extracted as described in [45]. Samples from two independent InlB extractions were analyzed by SDS-PAGE (data not shown). The resulting gel was fixed in a solution of methanol and acetic acid (10% and 7% v/v, respectively) for 20 min with gentle shaking, washed with dH_20 and stained with Sypro™ Ruby Protein Stain (Life Technologies, Carlsbad, CA, USA) overnight at room temperature with gentle shaking. In the morning, the gel was washed twice with the methanol and acetic acid fixative described above, then imaged and analyzed using a Fluor-S™ scanner (BioRad, Mississauga, ON, Canada) with PDQuest™ software. InlB bands were quantified as a percentage of total protein per sample. Total protein content of InlB extractions was determined using Coomassie Plus (Bradford) Protein Assay (Pierce Protein Research, Rockford, IL, USA). The assay was performed in quadruplicate, and average values taken. Concentrations of InlB were calculated by applying the percent InlB values obtained from PDQuest™ software to the Bradford assay results.

Clear, 384-well polystyrene Maxisorp™ (Thermo Fisher Scientific, Waltham, MA, USA) plates were coated with two-fold serial dilutions of extracted InlB in 1x PBS (pH 7.4, 25 µL), from 100 ng to 0.1 ng in quadruplicate. Plates were incubated overnight at 4 °C. The following morning, plates were rinsed with 1x PBS (pH 7.4, 115 µL) and blocked with 115 µL StartingBlock™T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hr at room temperature. Plates were rinsed again with 1x PBS (pH 7.4, 115 µL) and incubated with 50 ng R303 (biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer's instructions) diluted in 1x PBS (pH 7.4, 25 µL) for 1 hr at room temperature, followed by 5 washes with 0.1%-PBST (115 µL). Plates were incubated with 25 µL streptavidin conjugated to HRP (GE Healthcare, Baie d'Urfé, QC, Canada) used at a 1:1000 dilution in 1x PBS (pH 7.4) for 1 hr at room temperature, washed 5 times with 0.1%-PBST (115 µL) and developed with 25 µL TMB peroxidase substrate (KPL, Gaithersburg, MD, USA) for 30 min at room temperature. Assay development was stopped with phosphoric acid (1M, 25 µL), and the microtiter plate read at 450 nm on a MultiSkan FC spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). This experiment was independently repeated three times.

x) Panning of LAC naïve library against whole cells

Listeria monocytogenes, *Salmonella typhimurium*, and *E. coli* TG1were cultured at 37 °C, 250 rpm to an OD_{600nm} of 0.5. Cells were pelleted at 2800 × g for 15 minutes at room temperature using a Sorvall 5B Plus floor centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and pellets were washed once with 1x PBS. Cell pellets were resuspended and incubated for 30 min at room temperature in 5 mL 5% (v/v) formaldehyde for *L. monocytogenes* and in 2% (v/v) formaldehyde for *S. typhimurium* and TG1. Cells were pelleted after incubations and washed three times with 10 mL 1x PBS. Following the final wash, cell pellets were resuspended in 1x PBS to the original culture volume to try and establish the same cell density in each of the samples. A small aliquot of

cells was plated onto appropriate agar medium to ensure that cells were not viable before proceeding to panning.

Three rounds of panning against each species were carried out in solution. Before each round, the LAC phage library (100 μL) was incubated overnight at 4 °C, rotating with 100 μL of blocking agent in a round-bottomed tube. The next morning, the solutions were centrifuged for 10 min at maximum speed in a microfuge. The supernatants were collected and 500 μL of formalin-fixed cells were added and this solution was incubated overnight in round-bottomed tubes at 4 °C, rotating. The next morning, cells were pelleted at 5000 rpm in a microfuge. Pellets were washed three time with 0.1%-PBST followed by a final wash with 1x PBS. Cell pellets were resuspended in 100 μL 1x PBS and 1 mL of TG1 cells cultured to an OD_{600nm} of 0.3-0.5 was added directly to the suspension and incubated for 30 min at 37 °C, without agitation. The infected cells were then added to 9 mL of 2YT/Tet and the cultures were incubated overnight at 30 °C, 250 rpm. Phage particles were precipitated in PEG-NaCl [43] and resuspended in 1x PBS.

xi) Whole cell ELISA to assess LAC phage binding following panning

Wells of Maxisorb ELISA 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 100 μL of formalin-fixed cells and incubated overnight at 4 °C. The next morning, cell solution was removed and wells were washed once with 300 μL of 1x PBS. Wells were then blocked with 300 μL of StartingBlock™T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for one hour. Wells were washed once with 300 μL of 1x PBS. Two-fold serial dilutions of amplified, precipitated LAC phage following round 1 and round 3 of panning were added to wells and incubated at room temperature for 1 hour. Wells were washed four times with 300 μL 0.1%-PBST. An anti-M13 HRP conjugated antibody (Abcam, Cambridge, MA, USA)

was added at a 1:5000 dilution (100 μL per well) and incubated at room temperature for one hours. Wells were washed four times with 0.1%-PBST. TMB peroxidase substrate (KPL, Gaithersburg, MD, USA) was then added to each well (100 μL) and colour development was allowed to proceed for two to five minutes at which time 50 μL of 1 M H_2SO_4 was added. Absorbance was read at OD_{450nm} using a Multiscan FC plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Raw absorbance data were graphed versus phage dilution.

Results

I) Secondary structure analysis of recombinantly expressed Internalin A and Internalin B fragments

Antigens to be used in panning and/or VHH characterization studies were analysed using far-UV CD spectroscopy to assess if the proteins were folded. InlA-LRR was chosen as a target antigen to assess VHH cross-reactivity to a related Listeria invasin. Spectra collected at 20 °C and at 95 °C for both antigens were compared (Figure 1). The data indicated that both InlA (Figure 1A) and InlB (Figure 1B) fragments were folded. For InlB-LRR, the spectrum recorded at 20 °C was in agreement with published findings [34] . For InlA-LRR, it was observed that the protein lost secondary structure as it was heated, implying that the protein was folded (Figure 1A). We were not able to compare the spectrum for InlA with published data [46] as the construct we used was a fragment of the wild-type protein. However, based on its spectrum, the InlA fragment is showing the expected beta-strand character in a scan carried out at 20 °C. InlB-LRR was used in panning rounds and in VHH characterization studies whereas InlA-LRR was used only in VHH characterization studies..

II) Isolation of high affinity binders to Internalin B from camelid libraries

Four rounds of panning using the naïve LAC library were carried out to isolate high-affinity VHH candidates that could bind to the recombinant InlB-LRR protein. Clones obtained in round (II) were expected to be minimally enriched for antigen-specific VHH coding sequences, and since antigen specificity and genetic diversity were desired, colonies corresponding to eluted phage clones were picked from each of round (III) and (IV). VHH coding sequences from round (III) were expected to be less enriched, but more diverse, while coding sequences from round (IV) were expected to be more enriched and comprised of unique sequences [44]. All of the clones screened for binding to InlB-LRR were also screened for binding to blocking buffers used in the panning procedure to eliminate clones that were eluted but not InlB-LRR-specific. Negative controls were used to assess possible non-specific binding of anti-M13 antibody conjugated to HRP. An average A_{450nm} value was taken from four negative control wells per plate, and used to determine a level of background. A clone giving an A_{450nm} signal five times above background was deemed a positive binder. Using this criterion, all clones screened were positive for InlB-LRR binding. Single data points were obtained for each clone screened (data not shown). Uniform binding to InlB-LRR is seen in both round (III) and (IV), although A_{450nm} values for round (IV) binders appear to be on average, higher than those of round (III). However, this difference is most likely associated with ELISA development time rather than an indication of having higher affinities for InlB-LRR within the VHH-expressing phage pool. This is corroborated by coding sequence analysis, which shows that the majority of round (III) and (IV) clones were the same (data not shown).

Three rounds of panning were carried out with the immune library using a similar protocol as that used in the naïve library panning. Monoclonal phagemid ELISAs were performed to identify potential binders following sequencing analysis (data not shown). Two binders, Imm-R3-04 and Imm-R3-57, were isolated from a screening of nine unique clones. The remaining seven clones exhibited strong binding to blocking reagent and were therefore excluded from further analysis.

From the immune and naïve library panning campaigns, a panel of VHH clones that bound InlB-LRR were selected (Figure 2).

III) Binding and kinetics analyses of VHH clones using SPR

The panel of VHHs isolated from both libraries were expressed as monomers and biophysically characterised using surface plasmon resonance (SPR) (Figure 3). All VHHs listed in Table 1, with the exception of R3-26, displayed a monovalent interaction with InlB-LRR and fit the Langmuir-equation based 1:1 binding model (Figures 3A, 3B and 3D) [47]. R3-26 showed a slight bi-phasic binding at higher antibody concentrations which resulted in a poor fit for a 1:1 binding model. The bi-phasic binding is thought to be a result of non-specific interactions which occur at higher concentrations. The K_D value for the interaction between R3-26 and immobilized Internalin B was derived using the non-linear least squares fitting analysis [48]. This analysis allows the determination of the K_D value but not the k_{on} and k_{off} values;hence these kinetic constants are not reported in the case of the R3-26 VHH. Although the Imm-R3-04 clone was identified as a binder by phagemid ELISA, the monomeric form of this VHH did not exhibit binding to InlB-LRR in SPR analysis.
Preliminary epitope binning was carried out by binding the highest affinity binder, R3-03 at 50 x K_D, to immobilized InlB-LRR, followed by injection of a second VHH. A signal increase would result from the second VHH binding to a non-overlapping epitope. For all VHHs tested, no significant signal increase was observed, implying that the panel of VHHs bind to the same or overlapping epitope found on InlB-LRR. Figure 3E is a representative example using the Imm-R3-57 VHH.

IV) Antigen cross-reactivity assessment

To determine if the isolated antibodies specifically bound to InlB-LRR without cross-reacting to other *Listeria* LRR containing proteins, the related InlA-LRR protein was used as a target in SPR-based studies. The R3-03 and Imm-R3-57

VHH clones, representing the naïve library and immune library binders, were assessed. Both bind immobilized InlB-LRR with relatively high affinity (Table 1) but do not bind to immobilized InlA-LRR. Since no signal was observed when R3-03 was injected over the InlA-LRR surface, the surface was inferred to be free of bound VHH, and the surface was used again for Imm-R3-57 injection.

V) Recognition of biologically derived antigen by VHH clones isolated from the naïve library

InlB derived from the surface of wild-type *Listeria monocytogenes* EGD serovar 1/2a using a Tris-based extraction protocol [45] was used in ELISA experiments to determine if the anti-InlB-LRR VHHs isolated from the naïve library panned against a recombinant antigen could also bind the natural target (Figure 4). Extract from the *inlB* knockout *Listeria* cell line (ΔinlB) and the *inlA* knockout (ΔinlA) were used as controls. The R3-03 clone, which had the highest affinity for InlB-LRR as determined by SPR analysis, reproducibly bound immobilized, wild-type InlB and ΔInlA extracts. No binding to the immobilized extract from the ΔinlB cells was observed. To ensure that reproducible amounts of target protein were being added to the ELISA wells, the extraction process was carried out independently, four times. The extracts were resolved in 12.5% SDS-PAGE gels and the gels were Coomassie stained. It was found that a consistent yield of protein was obtained amongst the trials. Densitometry analysis determined that InlB constituted approximately 20% of the proteins extracted from the bacterial surface using this extraction protocol (data not shown). The VHH clone derived from the immune library was not assessed at the time the naïve library VHH clones were assessed due to timing. However, since Imm-R3-57 does bind to InlB-LRR and was shown to likely recognize an overlapping epitope in competition with R3-03, it is reasonable to assume that Imm-R3-57 would also recognize the biological antigen.

Discussion

To develop antibody-based reagents for potential use in bacterial detection strategies, a naïve and an immune M13 phage-based camelid library were used in a panning strategy against the *Listeria* surface virulence factor, InlB. Panning against an InlB-LRR recombinant construct with the naïve library resulted in successful isolation of four distinct clones whose sequences were enriched following four rounds of panning. All four clones possess dissociation constants in the low nanomolar (nM) to high picomolar (pM) range. Two of the four, R3-03 and R3-30, possess binding affinities in the pM range with a fast on-rate and a slow-off rate. Construction and panning of a library derived from immunizing a llama with the recombinant InlB-LRR construct, resulted in the identification of two potential binders following three rounds of panning. Imm-R3-57 was analysed in Biacore experiments and found to have similar binding affinity and binding kinetics as the VHHs isolated from panning with the naïve library. Imm-R3-04, though it exhibited binding in a phagemid ELISA assay, did not bind to the InlB-LRR construct in SPR experiments. Although similar behaviour has been observed for other VHH clones targeting other protein antigens, whereby a positive phage ELISA result does not correlate with SPR results, it is not clear what causes this (observation in our lab).

Since it was possible to isolate VHH binders displaying low nM to high pM binding affinities from a naïve library, it is evident that the naïve LAC phage library repertoire contains affinity matured HCAb sequences, as one or more of the animals from which the B-lymphocytes were derived had been previously exposed to this *Listeria* antigen. This can be advantageous as it may not be necessary to immunize animals and construct an immune library for the express purpose of isolating high affinity binders to this antigen or to antigens expressed by environmental pathogens. Furthermore, it may be possible to use the naïve library in panning experiments to isolate high-affinity antibodies against antigens from other environmental or foodborne pathogens. Preliminary experiments using the LAC phage library to pan against other environmental bacterial pathogens demonstrates that specific binders recognizing cell membrane

proteins can be isolated (Figure 5). This could potentially decrease both the cost and effort of isolating high affinity VHHs targeting such bacterial pathogens.

As we have been able to isolate relatively high-affinity anti-InlB binders from a naïve phage library, it is plausible that the llama which was immunized with the InlB-LRR recombinant antigen may have already encountered the same antigen through natural routes. Therefore, the binders isolated from the immune library may have been present at the pre-immune stage of the immunization protocol. When pre-immune and immune sera immunoglobulin levels were fractionated as described [49] and assayed for binding to InlB-LRR by ELISA, the pre-immune serum already contained an HCAb population which bound to the antigen (data not shown). Furthermore, these pre-immune levels did increase following immunization, so it is difficult to assess from which population, i.e. pre-immune or immune, the binders were isolated. There are conflicting reports on whether or not further somatic hypermutation occurs in memory B-cells upon antigen re-challenge [50,51], so we cannot make conclusions regarding the origins of these VHHs isolated from the immune phagemid library.

SPR epitope binning data demonstrated that all assessed VHHs recognize the same or an overlapping antigenic epitope found on InlB-LRR. This may indicate that the natural immune response in the animal is directed to an immuno-dominant epitope. However, the *in vitro* panning approach taken to isolate binders may also affect the outcome as there have been instances where antigen-immobilized panning and soluble-antigen panning have produced non-overlapping binders [52].

To determine if the VHHs isolated from both pannings demonstrated specificity, a related protein, InlA, was used as the target antigen in SPR analysis. Since the binding region on InlB-LRR was determined to be the same or overlapping for all VHHs, R3-03, the VHH exhibiting the highest affinity from the panel of naïve library binders, was chosen as a representative binder. Far UV CD spectroscopy

of both InlA-LRR and InlB-LRR antigens used in this study showed the characteristic spectra associated with these proteins, demonstrating that the recombinant proteins are folded and functional. Surprisingly, although both Internalin fragments possess LRR-repeats and therefore, possible structurally cross-reactive epitopes, the InlB-LRR binders do not interact with InlA-LRR. However, it cannot be definitively stated that the InlB-LRR binders will not cross react with other *Listeria* Internalins or even with structurally related proteins expressed by other prokaryotic and/or eukaryotic species. These possibilities were not explored as they were beyond the scope of this study. The InlB-LRR epitope recognized by our panel of VHHs has not been mapped but work is underway to elucidate the interaction.

The secondary structure of InlB-LRR was confirmed using CD spectroscopy but it should be noted that the length of the LRR construct used in this study is 241 residues, whereas full-length InlB is 630 residues long. However, there is confidence that the construct is properly folded as its spectrum at room temperature agrees well with those of a 321 aa and a 248 aa construct [34]. Both of the latter constructs have almost overlapping spectra which also overlap with the 241 aa fragment spectrum. Regarding the InlA-LRR construct, it may be argued that the lack of cross-reactive binding may be due to non-functional protein. CD spectroscopy demonstrated that the protein does have the expected secondary structure, but due to lack of published spectra for the fragment utilized in these studies for comparison, we cannot definitively state that it is folded correctly. SPR binding experiments with E-cadherin, the natural target of InlA, or ideally, structure determination, may provide a more definitive answer. The fragment utilized in these studies is identical to the one used to determine the structure of the LRR domain of InlA [46]. Furthermore, the protein was expressed and purified using similar methodologies, so it is reasonable to believe that this recombinant fragment would be folded and functional.

Although the VHHs described in this study were isolated against a recombinant InlB-LRR construct, these antibodies also bind to biologically derived antigen, even in the presence of contaminating proteins. The signal increase at the higher concentrations of the ΔinlB negative control may be attributable to non-specific binding. The method used to extract biologically derived InlB does not extract the structurally-related invasin InlA, as it is crosslinked to the *Listeria* cell wall [32]. In order to confirm the specificity of the isolated VHHs, recombinant InlA-LRR was expressed and purified. The assessment of binding using biologically derived antigen confirms that an *in vitro* antibody library panning protocol using a recombinant fragment of the biological antigen is a viable approach to isolate high affinity antibody binders against the biological target. It is especially striking that the isolation of high-affinity VHH binders can be successfully carried out using a repertoire derived from a passively immunized camelid and panning against a recombinant fragment of the wild-type protein.

The R3-03 VHH contains a putative intera-CDR disulphide bond while the other three VHHs from the naïve library series do not. This extra disulphide bond may contribute to R3-03 having a 1000-fold higher affinity for InlB-LRR than R4-19. There is literature describing stabilization and increased binding affinity attributable to the presence of intra-CDR disulphide bonds in VHHs [53]. We speculate that this inter-CDR disulphide bond may function to stabilize the VHH paratope, perhaps allowing binding of epitopes inaccessible to VHHs lacking this extra disulphide bond. Work is underway to elucidate the binding mechanism of the isolated anti-InlB-LRR VHHs.

In this study, we have demonstrated that high affinity VHHs against a relevant environmental pathogen, i.e. *Listeria monocytogenes*, can be successfully isolated from not only immunized but also so-called naïve library sources. To the best of our knowledge, this is the first report of anti-InlB VHHs. Antibody repertoires from naturally immunized sources may be equally robust sources for isolation of high-affinity binders as from artificially immunized sources. In fact,

we have preliminary data indicating that such sources can be used to isolate specific binders to environmental pathogens of interest by panning against whole cells (Figure 5, results from *S. typhimurium* panning not shown). This may help to decrease costs associated with isolating antibodies against certain types of targets. Furthermore, these antibodies can be used as potential reagents in the development of detection technologies and perhaps even medical technologies.

Acknowledgements

We would like to thank Pascale Cossart at the Pasteur Institute for kindly providing us with the *Listeria monocytogenes* EGD serovar and the related knockout strains, Perry Fleming for arranging with Health Canada for import of the cells from the Cossart lab, Hiba Kandalaft for expertise and discussion regarding CD spectroscopy studies, Sonia Leclerc for carrying out DNA sequencing, NSERC and Sentinel Bioactive Paper Network for providing funding (grant number).

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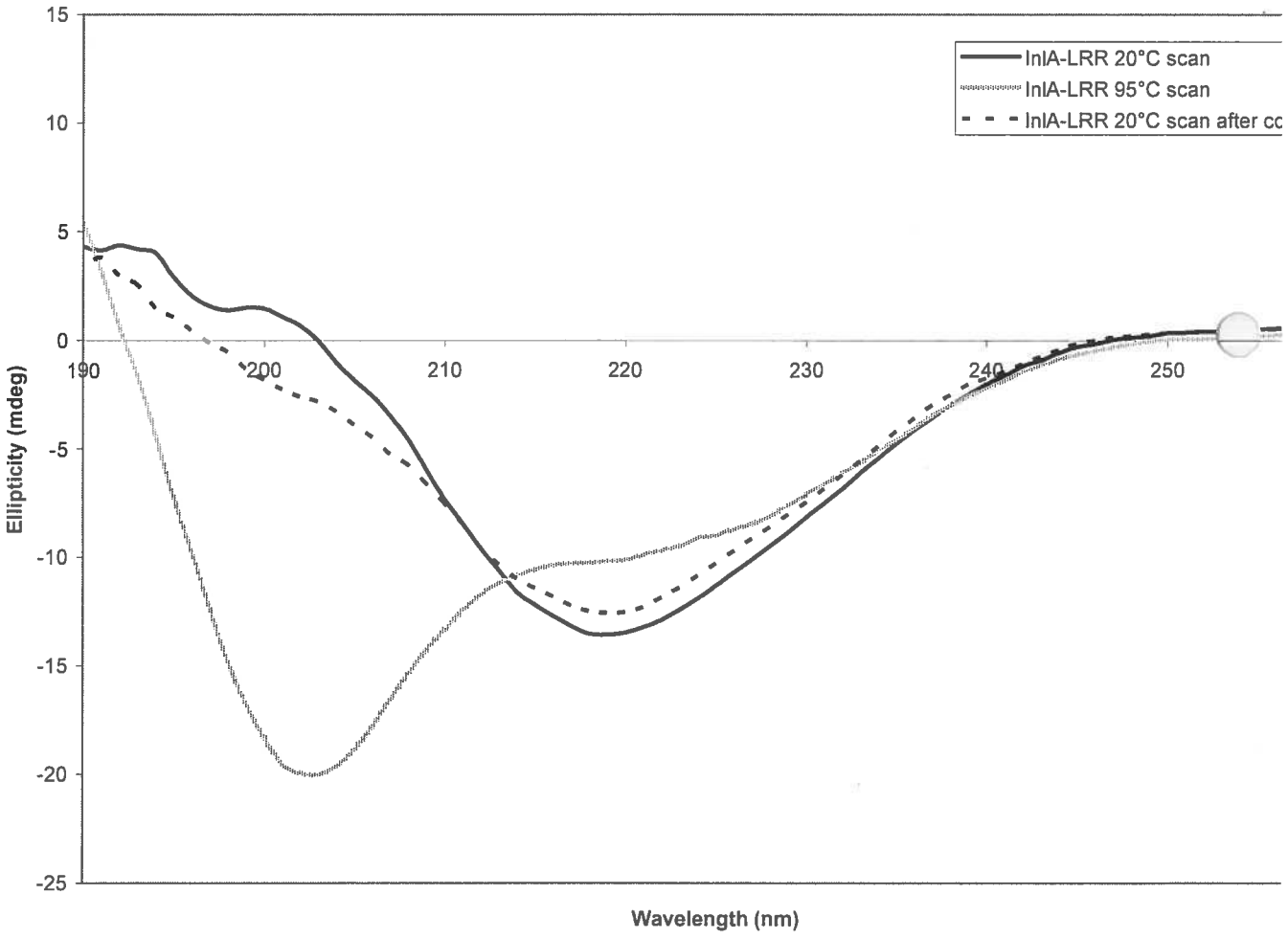
Figure 1 Far-UV spectra of Internalin A (upper panel) and Internalin B (lower panel) LRR fragments to assess proper folding of the recombinantly expressed antigens. The same aliquot was used in all three scan conditions for both proteins. Following a 20°C scan, the sample was heated to 95°C and then scanned and finally, cooled to 20°C and re-scanned. InlA-LRR was used at a concentration of 0.1 mg/mL and InlB-LRR was used at a concentration of 1.33 mg/mL.

A



B

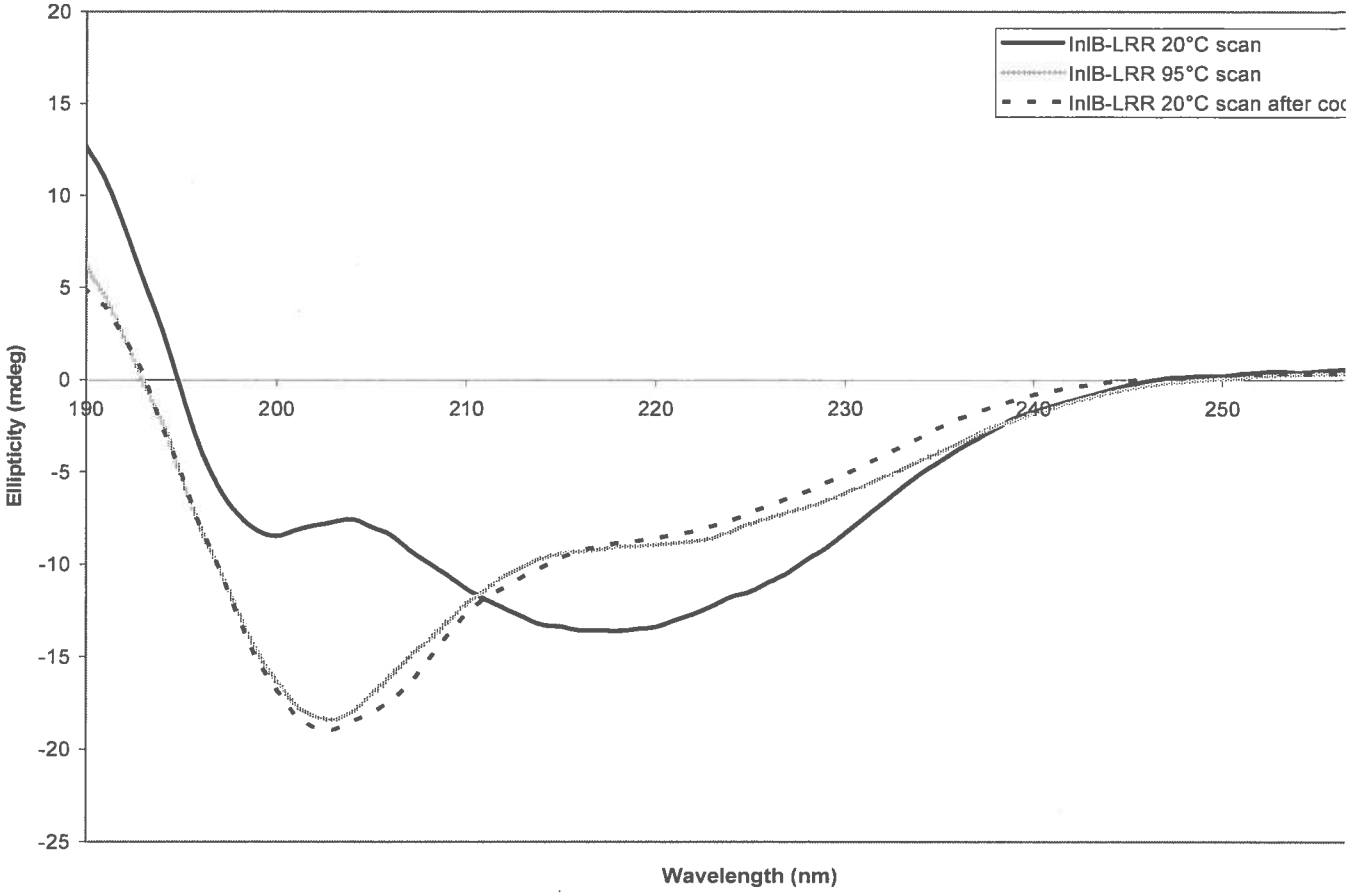

InlB-LRR 20°C scan
InlB-LRR 95°C scan
InlB-LRR 20°C scan after coo
Ellipticity (mdeg)
Wavelength (nm)
20
15
10
5
0
-5
-10
-15
-20
-25
190
200
210
220
230
240
250

Figure 2 Alignment of InlB-LRR-specific VHH coding sequences. Framework and CDR boundaries are in accordance with the Kabat database.

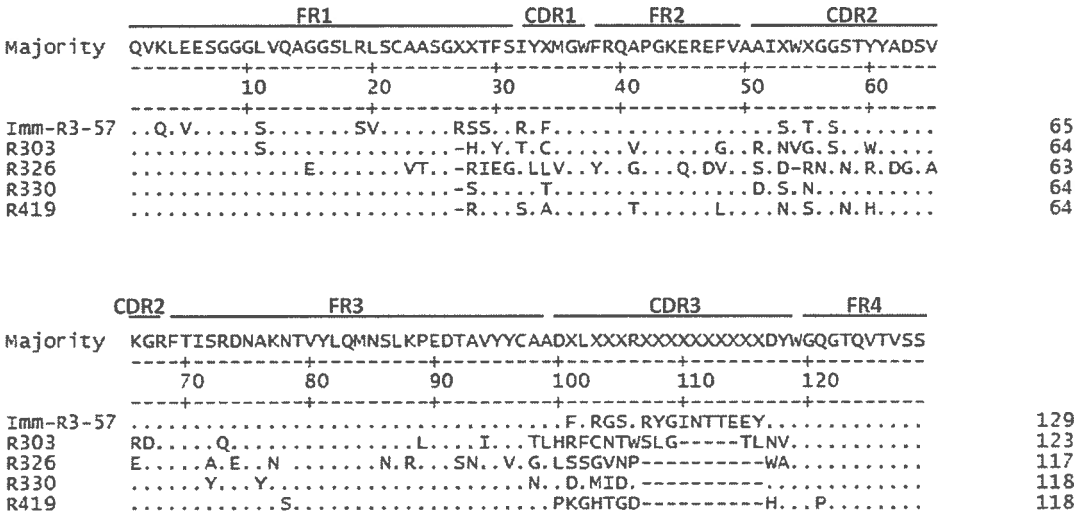
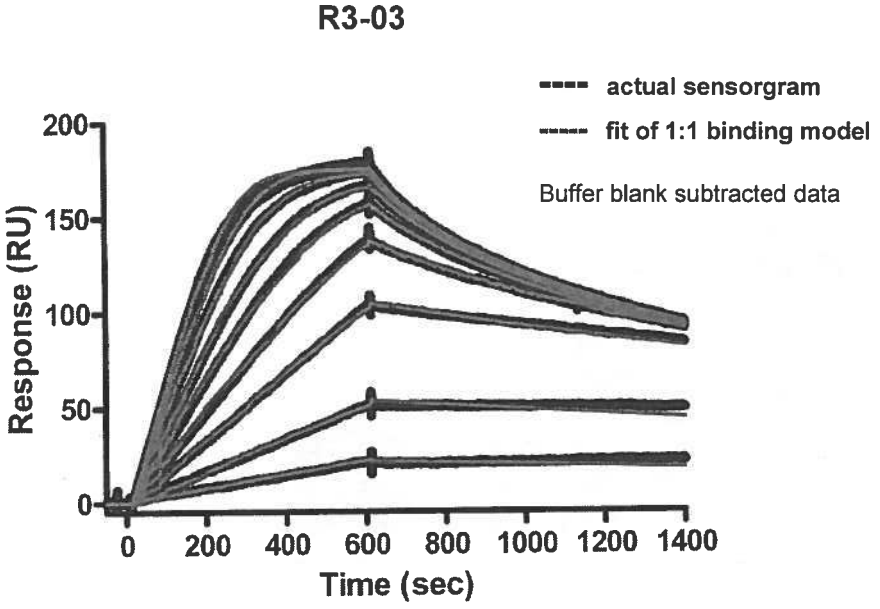
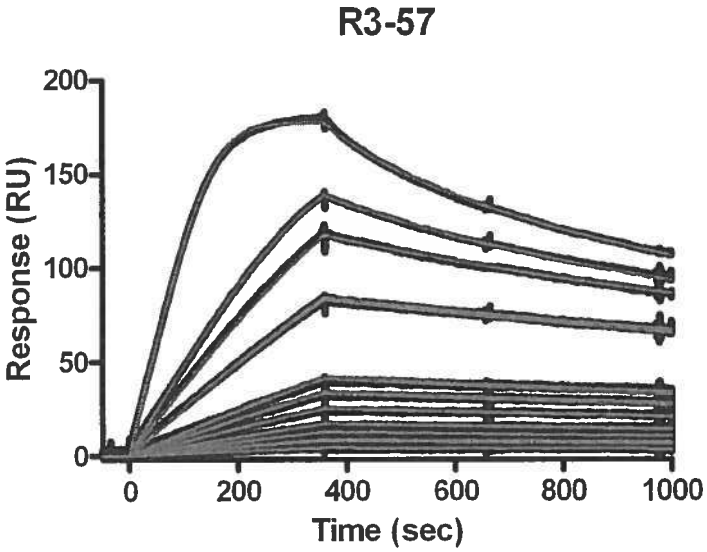


Figure 3 Evaluation of VHH clones binding to immobilized InlB-LRR using surface plasmon resonance. Clones screened in phage ELISAs using immobilized antigen were expressed and purified in monomeric format for use in binding assays. Low surface densities (theoretical R_{max} ~ 200 RU) of InlB-LRR cross-linked to CM5 sensorchips were prepared. VHH proteins were assayed individually at various concentrations (panel A to D R3-03 and R3-30, 2 nM to 0.1 nM; R3-26, 15 nM to 0.5 nM; R3-57 250 nM to 2.5 nM) to determine binding constants and binding kinetics listed in Table I (actual sensograms in black lines, fitted binding models in red lines). Buffer blank has been subtracted. Preliminary epitope mapping was carried out using competition analysis (panel E) between R3-03 (representative clone from LAC library) and R3-57. All VHH clones bind the same or overlapping epitope on InlB-LRR.

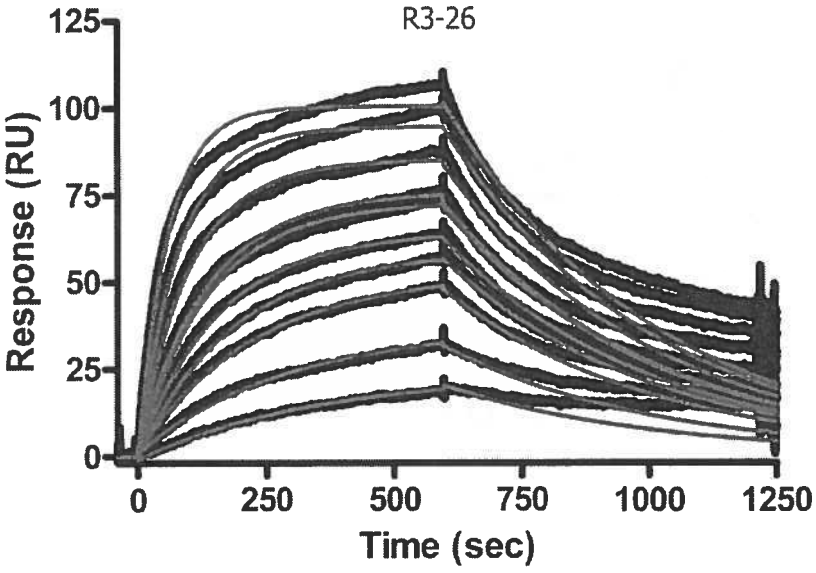
A



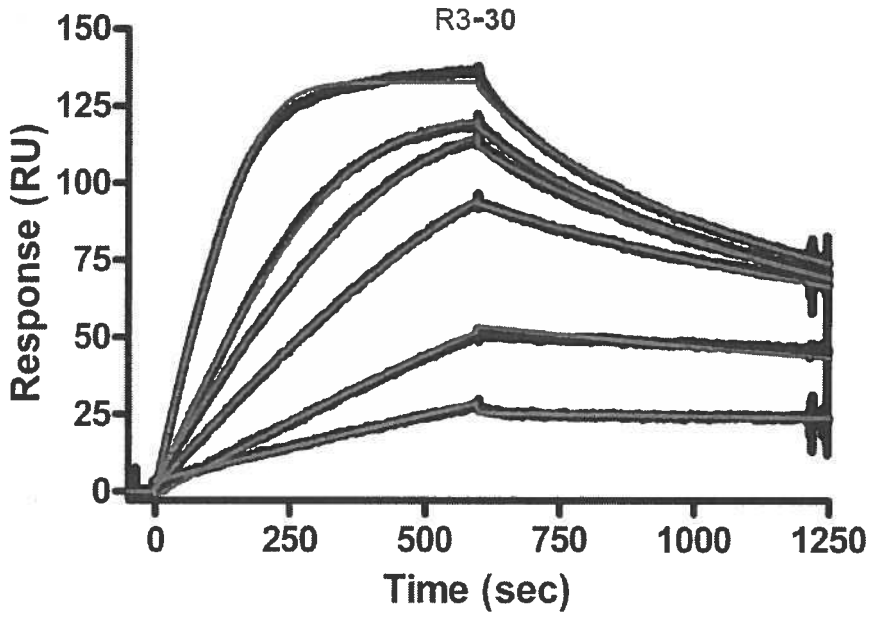
B


R3-57
Response (RU)
200
150
100
50
0
0
200
400
600
800
1000
Time (sec)

C


R3-26
Response (RU)
125
100
75
50
25
0
0
250
500
750
1000
1250
Time (sec)

D


R3-30
Response (RU)
150
125
100
75
50
25
0
0
250
500
750
1000
1250
Time (sec)

E

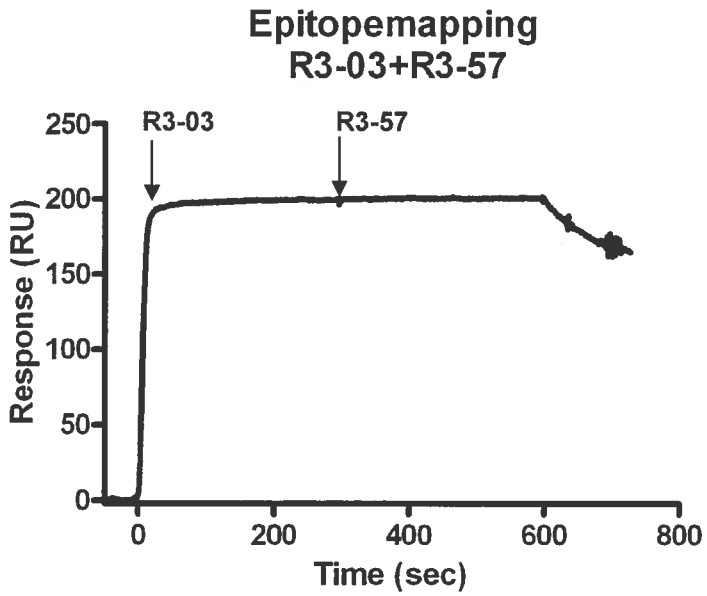

Epitopemapping
R3-03+R3-57
R3-03
R3-57
Response (RU)
250
200
150
100
50
0
0
200
400
600
800
Time (sec)

Figure 4 Recognition of biological antigen by R3-03 in ELISA. Proteins, stripped using a Tris buffer method, from the surface of Listeria strains grown in liquid culture were immobilized in ELISA plate wells. Biotinylated R3-03 was titrated into the wells following incubation with a blocking agent. Streptavidin-HRP was used to detect the bound VHH using standard ELISA colorimetric methodology. Representative results of three independent trials.

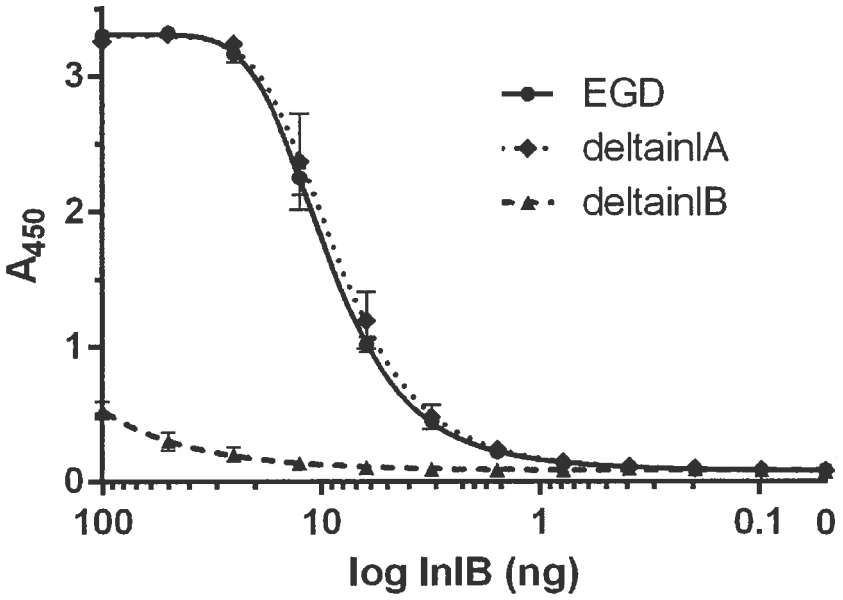
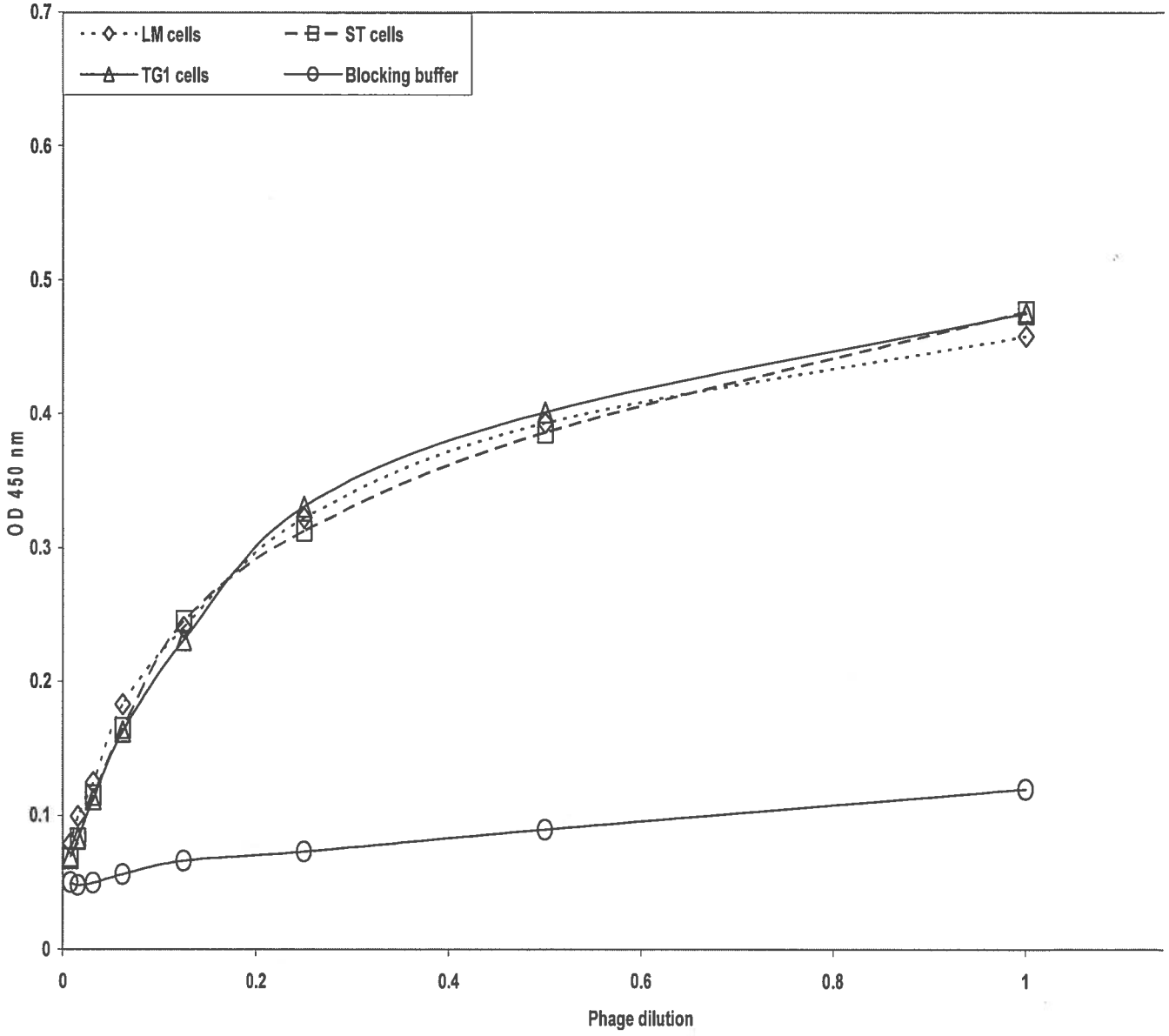


Figure 5 Preliminary ELISA results of amplified LAC phage following round1 (upper panel) and round three (lower panel) of panning using formalin-fixed whole cell targets. Cells were immobilized in plate wells and 2-fold serial dilutions of amplified, concentrated phage were added to wells. An anti-M13-HRP conjugated antibody was used to detect any bound phage. Wells incubated with blocking agent were used as negative controls for each dilution of phage. LM-*Listeria monocytogenes*, ST-*Salmonella typhimurium*, TG1- *Escherichia coli TG1*.



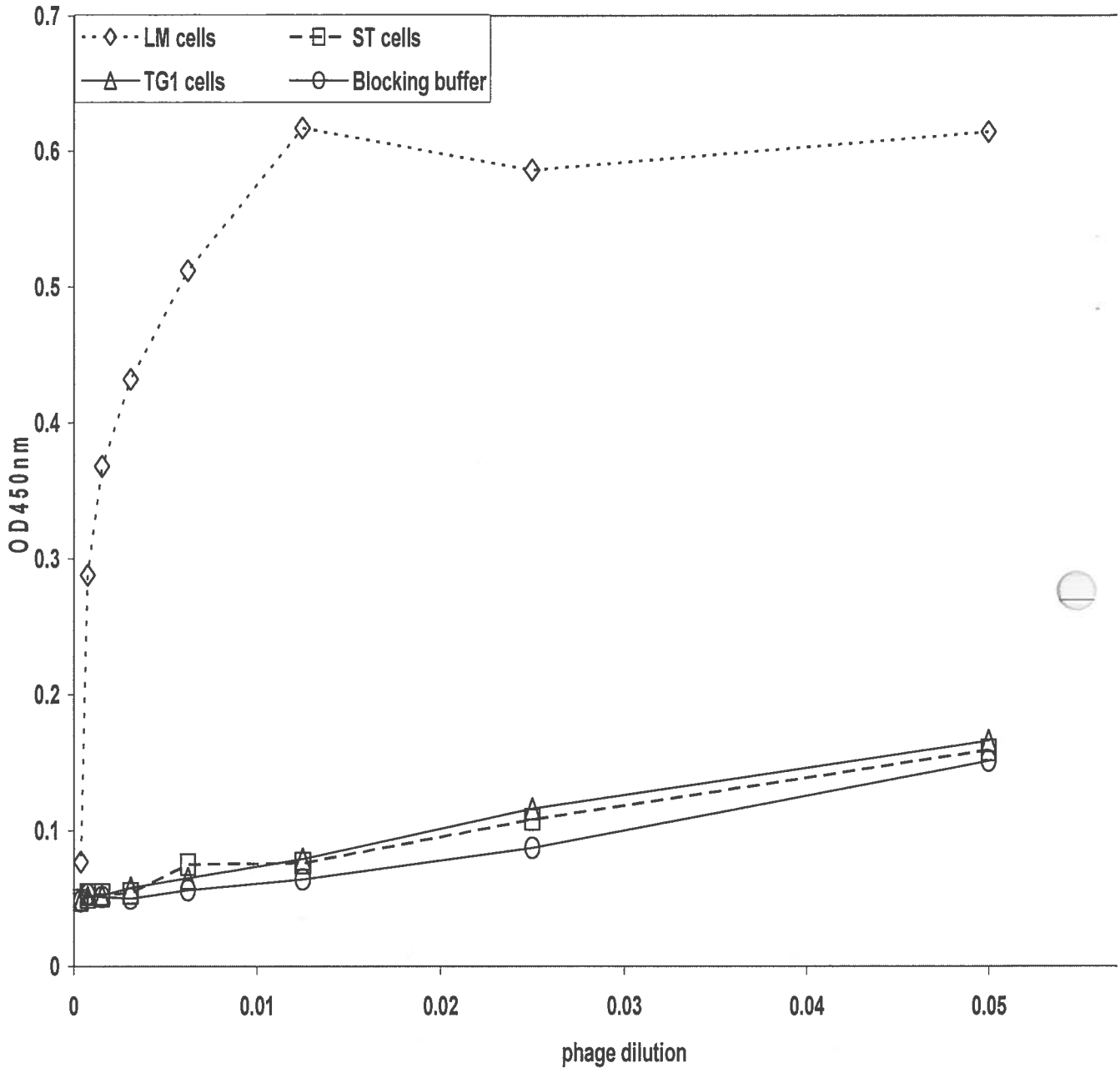

LM cells
ST cells
TG1 cells
Blocking buffer
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 450nm
0
0.01
0.02
0.03
0.04
0.05
phage dilution

Table 1 SPR data – Binding kinetics of anti-InlB VHHs

VHH	K_D (M)	K_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	χ^2
R3-03	1.5×10^{-10}	1.7×10^{7}	2.7×10^{-3}	1.45
R4-19	1.5×10^{-7}	1.9×10^{3}	3.0×10^{-4}	5.76
R3-26	3.9×10^{-9}	ND[a]	ND[a]	NA[b]
R3-30	1.3×10^{-10}	1.5×10^{7}	2.0×10^{-3}	1.00
Imm R3-57	9.4×10^{-9}	1.8×10^{5}	1.7×10^{-3}	2.15

[a]not determined [b]not applicable to type of analysis

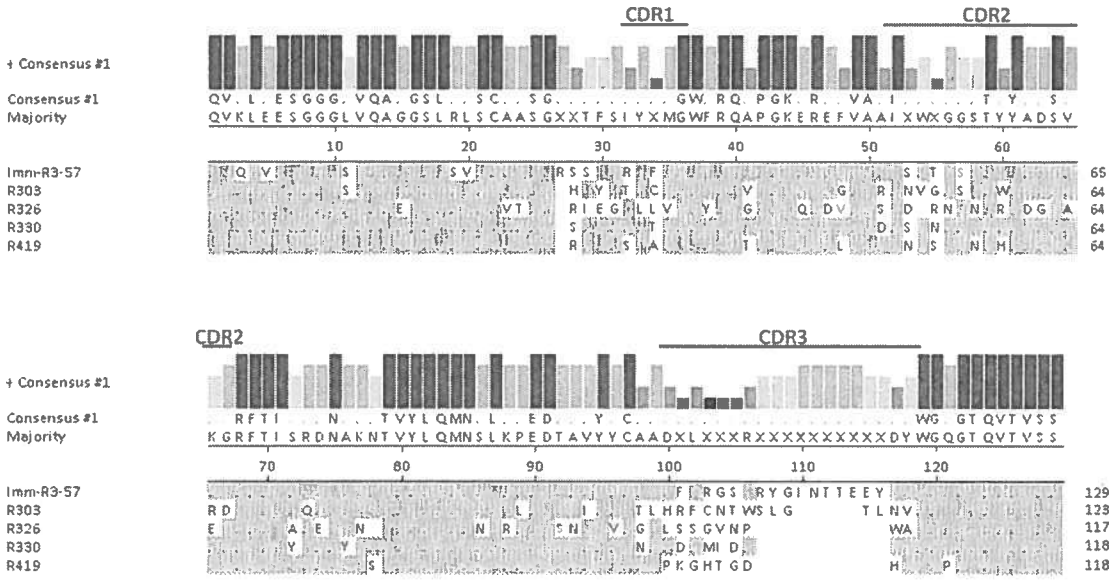
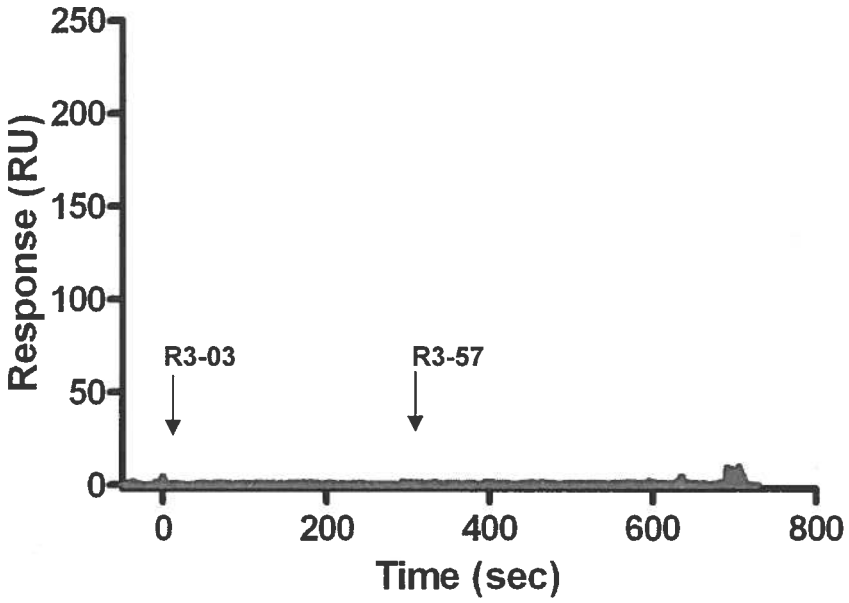

CDR1
CDR2
+ Consensus #1
Consensus #1
Majority
10
20
30
40
50
60
Imm-R3-S7
R303
R326
R330
R419
65
64
64
64
64
CDR2
CDR3
+ Consensus #1
Consensus #1
Majority
70
80
90
100
110
120
Imm-R3-S7
R303
R326
R330
R419
129
123
117
118
118

Figure 6 Evaluation of cross-reactivity of VHHs which recognize InlB-LRR. The R3-03 clone, the representative InlB-LRR binder isolated from the LAC library panning and the R3-57 clone from the immune library were evaluated. InlA-LRR was immobilized to low surface density (theoretical Rmax ~ 200 RU) on a CM5 sensorchip and VHH concentrations of 50-fold KD were used. The same surface was used to evaluate both VHH clones as R3-03 injection over the chip did not result in an increase in signal, i.e., no interaction of VHH with immobilized antigen.



R3-03 and R3-57 did not bind to the Internalin-A surface at concentrations 50xK_D.