

Supplemental Methods:

Bacterial strains and cloning

Streptomycin-resistant strain *Shigella flexneri* serotype 5a (M90T-Sm) was used (Onodera et al.). *Shigella* was routinely cultured in or on Trypticase Soy Broth (TSB), with or without 20 mg/mL agar and 0.01% Congo red. Tetracycline was used at a concentration of 5 µg/mL to select for the tetracycline resistance cassette (*tetRA*). Streptomycin (Sm) was used for selection at 100 µg/mL in MacConkey medium. To construct the *ospG::tetRA* mutant, *ospG* was deleted in M90T using lambda red recombination in a manner similar to that described in (Baba et al., 2006)). Oligonucleotides used in construction of the *ospG* mutant and for the complementation strategy are listed in Supplemental Table 1. The plasmid *tetRA*-pGEM was created using the tetracycline resistance gene *tetRA* from the DNA fragment TH2788 (Karlinsey, 2007). *TetRA* was first amplified by PCR using the primers tet 2F-PstI and tet 2R-PstI (Supplemental Table 1) and cloned into pGEM-T Easy using TA cloning. Using this plasmid as template, *tetRA* was amplified using the primers T3 LR1R HindIII and T7 LR1F KpnI and cloned into pGEM-T Easy using TA cloning, creating the plasmid "*tetRA*-pGEM." The primers T3 LR1R HindIII and T7 LR1F KpnI facilitated the addition of FRT sequences to *tetRA*, as well as sequences known as "P1" and "P2". A linear DNA fragment used to replace *ospG* on the pWR100 virulence plasmid was created by PCR from the *tetRA*-pGEM template using primers containing the P1 and P2 sequences, as well as ~50 bp of homology to the sequence flanking *ospG*. In the forward primer, this region of homology began 47 bp upstream of the *ospG* start codon and ended directly after. The reverse primer started 29 bp downstream of *ospG* and ended 21 bp into the coding sequence. The knock-out cassette was purified using a Qiagen PCR purification kit.

To establish a parent strain for lambda red recombination, pKD46 was transformed into electrocompetent M90T-Sm (Datsenko and Wanner, 2000) followed by selection using 100 µg/mL ampicillin at 30°C incubation. To prepare electrocompetent cells, a 2 mL culture of M90T-Sm pKD46 was grown overnight, then sub-cultured 1:100 in 50 mL of medium containing 1% L-arabinose the next morning. Sub-cultures were grown to an OD₆₀₀ between 0.4 and 0.6, at which time the bacteria were pelleted and resuspended twice in 25 mL ice cold water. All centrifugation steps were performed using an Eppendorf 5810R centrifuge with an A-4-81 rotor at 3,220 g and 4°C for 10 minutes, unless otherwise stated. The bacteria were then pelleted a fourth time and resuspended in approximately 500 µL of water. 50 µL of the resuspended bacteria were added to a 2 mM electroporation cuvette along with 15 µL of purified knock-out cassette. The bacteria were electroporated, then allowed to recover for 2 hours in 1 mL of TSB at 37°C while shaking at 200 RPM. The bacteria were then pelleted at 2,300 g for 5 minutes at room temperature and plated on a TSB Congo red plate containing tetracycline and grown overnight at 37°C to select for integration of the knock-out cassette. Loss of pKD46 was selected for by growth on TSB Congo red plates at 37°C. Integration of the knock-out cassette at the desired location was confirmed by PCR using primers P1 and a primer downstream of *ospG*.

For complementation studies, *ospG* was introduced into the *ospG::tetRA* virulence plasmid under control of the constitutive *lac* promoter. Plasmid pM90SIRC was created by introducing a DNA fragment corresponding to 71414-71913 of pWR100 into the suicide integration vector pJQ200 (Quandt and Hynes, 1993). This region of pWR100 contains no gene coding regions and represents a "safe insertion region" as defined by Isaaks (Isaacs et al., 2011). Insertion of gene encoding DNA into this safe region does not alter *Shigella* fitness, ability to invade epithelial cells, or alter T3SS activity *in*

vitro (data not shown). Wild type *ospG* (*ospG*^{wt}) or *ospG* containing substitutions G81R and C127R (*ospG*^{mut}) or K53M (*ospG*^{CD}) were amplified and cloned into pBluescript to create plasmids pBS-*ospG*^{wt}, pBS-*ospG*^{mut}, and pBS-*ospG*^{CD}, respectively. pBS-*ospG*^{wt}, pBS-*ospG*^{mut}, and pBS-*ospG*^{CD} were digested with BamHI and XhoI and were introduced into pM90SIRC to create plasmids pSIRC-*ospG*^{wt}, pSIRC-*ospG*^{mut}, and pSIRC-*ospG*^{CD}, which were then introduced into *E. coli* strain S17-1λ pir. *Shigella ospG::tetRA* was mixed with S17-1λ pir pM90SIRC in approximately a 1:1 ratio, spotted on TSB and allowed to incubate at 37°C for 5 hours. The mix of bacteria was then plated on TSB plates with tetracycline and gentamycin (to select for *Shigella* carrying pSIRC plasmids) and incubated at 37°C overnight. Colonies were patched onto TSB Congo red plates containing tetracycline and gentamycin. Integration of *ospG*^{wt}, *ospG*^{mut}, and *ospG*^{mut} were confirmed using oligonucleotides specific to *ospG* and SIRC.

Analysis of secreted effectors in vitro

Bacterial strains were grown to mid-log phase, and approximately 4×10^8 bacteria were pelleted at 3,220 g for 10 minutes and resuspended in one mL of PBS. Congo red was added to a final concentration of 425 µg/mL and the bacteria were incubated at 37°C for 30 minutes. Bacteria were pelleted at 3,000 g for 10 minutes, then the supernatant was collected and filtered through a 0.45 µm filter. Supernatant samples were mixed with SDS sample buffer in a 3:1 ratio of sample to buffer and boiled for five minutes before gel loading. All samples were run on 10% SDS-PAGE gels. Gels were stained using a silver stain kit from BioRad (catalogue number 161-0449).

Mouse infection

Five to eight week-old female BALB/c mice were obtained from Charles River (St Constant, QC, Canada). Six- to eight-week-old mice were used for all the experiments. They were acclimatized in the Health Centre animal facility for 1 week before experimentation. Experiments were undertaken in the IWK Health Centre under the approval of the University Committee on Laboratory Animals, Dalhousie University. All animals were housed in rooms maintained at 21°C with 12 hour dark-light cycles, and had free access to food and water. The results of all animal studies are reported in accordance with the ARRIVE guidelines (McGrath et al., 2010). A minimum of 10 mice were used in each experimental group. Mouse infections were performed as described by Martino (Martino et al., 2005). Mice were given 5 g/L streptomycin in their drinking water for 2 days prior to infection, and continued to drink the antibiotic for the remainder of the experiment. Mice were taken off their food 6 hours before oral infection. The night before oral infections, a single *Shigella* colony was picked from a Congo red plate and grown overnight at 37°C in a 5 mL TSB culture. In the morning, this growth was subcultured 1:10 into 9 mL of fresh TSB (10 mL total), and grown for approximately 3 hours at 37°C. The 10 mL culture was centrifuged at 3,220 g for 10 minutes, and resuspended in 1 mL of sodium bicarbonate (1.4%). Mice were infected by oral gavage with 0.1 mL of this *Shigella* suspension, resulting in a dose of approximately 10^8 bacteria. The mice were monitored for either 3 or 5 days with daily assessments on severity of clinical illness and collection of fecal samples to determine bacterial burden. Clinical illness was scored based on appearance (grooming, urine staining, and coat quality), behavior (response to stimulus, posture, lethargy), appetite, hydration, temperature, and weight loss. Mice that had lost greater than 20% of their initial body weight or those that did not respond to stimulus were euthanized.

On the final day of the experiment, the mice were placed into a surgical plane of unconsciousness with isoflourine and euthanized by cervical dislocation and cardiac puncture. Blood collected from the cardiac puncture was left at room temperature to coagulate then centrifuged at 2,300 g for 15 minutes. The blood serum was collected and frozen prior to further analysis. The colon, cecum, liver, and spleen were surgically removed. The colon and cecum were flushed with ice cold PBS. The colon was cut along the marginal artery to create a single layer. This layer was then cut longitudinally again to create two halves. One half of the colon was Swiss rolled from the proximal to distal end and placed in a histology cassette in 10% Formalin overnight along with half the spleen and the cecum. The histology samples were then sent to the IWK's Histology Department to be embedded in parafin and to have slides prepared. The slides were viewed and assessed, in a blinded fashion, for signs of inflammation and destruction of mucosal epithelium.

Feces were collected daily from each mouse, weighed, homogenized in saline solution, and serially diluted onto MacConkey medium supplemented with Sm (100 ug/mL). Bacterial counts are reported as colony forming units per gram of feces. Livers and half spleens were weighed, homogenized and diluted into a volume of cold saline equal to 5 times the weight, serially diluted, and plated onto MacConkey medium supplemented with Sm. Content of the cecum was collected and serially diluted into ice cold PBS, then plated onto MacConkey medium supplemented with Sm.

Data analysis

A non-parametric Kruskal-Wallis test was used for statistical analysis of bacterial counts in feces and organs with a p value <0.05 for significance, followed by a Dunn's multiple comparison (Sellge et al., 2010). Survival curves were analyzed using a Log-rank (Mantel-Cox) test with significance defined as a p value <0.05.

References:

- Ashkenazy H., Erez E., Martz E., Pupko T., Ben-Tal N. (2010) ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res* 38:W529–533
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Sys. Biol.* **2**: 1-11.
- Datsenko, K. A., and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**: 6640-6645.
- Dou, H., Buetow, L., Sibbet, G. J., Cameron, K., and Huang, D. T. (2012). BIRC7–E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. *Nat. Struct. Mol. Biol.* **19**: 876-883.
- Isaacs, F. J., et al. (2011). Precise manipulation of chromosomes in vivo enables genome-wide codon replacement. *Science* **333**: 348-353.

Kamaduri, H.B., Souphron, J., Scott, D.C., Duda, D.M., Miller, D.J., Stringer, D., Piper, R.C., and Schulman, B.A. (2009) Insights into Ubiquitin Transfer Cascades from a Structure of a UbcH5B~Ubiquitin HECT(NEDD4L) Complex. *Mol. Cell* **36**:1095-1102.

Karlinsey, Joyce E. (2007). λ -Red Genetic Engineering in *Salmonella enterica* serovar Typhimurium. *Meth. Enzymol.* **421**: 199-209.

Martino, M. C., Rossi, G., Martini, I., Tattoli, I., Chiavolini, D., Phalipon, A., Sansonetti, P. J., and Bernardini, M. L. (2005). Mucosal lymphoid infiltrate dominates colonic pathological changes in murine experimental shigellosis. *J. Infect. Dis.* **192**: 136-148.

McGrath, J. C., Drummond, G. B., McLachlan, E. M., Kilkenny, C., and Wainwright, C. L. (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. *Br. J. Pharmacol.* **160**: 1573-1576.

Onodera, N. T., Ryu, J., Durbic, T., Nislow, C., Archibald, J. M., and Rohde, J. R. (2012). Genome Sequence of *Shigella flexneri* Serotype 5a Strain M90T Sm. *J. Bacteriol.* **194**: 3022-3022.

Plechanovová, A., Jaffray, E. G., Tatham, M. H., Naismith, J. H., and Hay, R. T. (2012). Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. *Nature* **489**: 115-120.

Quandt, J., and Hynes, M. F. (1993). Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* **127**: 15-21.

Sellge, G., Magalhaes, J. G., Konradt, C., Fritz, J. H., Salgado-Pabon, W., Eberl, G., Bandeira, A., Di Santo, J. P., Sansonetti, P. J., and Phalipon, A. (2010). Th17 cells are the dominant T cell subtype primed by *Shigella flexneri* mediating protective immunity. *J. Immunol.* **184**: 2076-2085.