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Supplementary appendix 1

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Bernshtein B, Kelly M, Cizmeci D, et al. Determinants of immune responses predictive of protection against shigellosis in an endemic zone: a systems analysis of antibody profiles and function. *Lancet Microbe* 2024. https://doi.org/10.1016/S2666-5247(24)00112-5

Appendix 1

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Methods

Procedures

Luminex

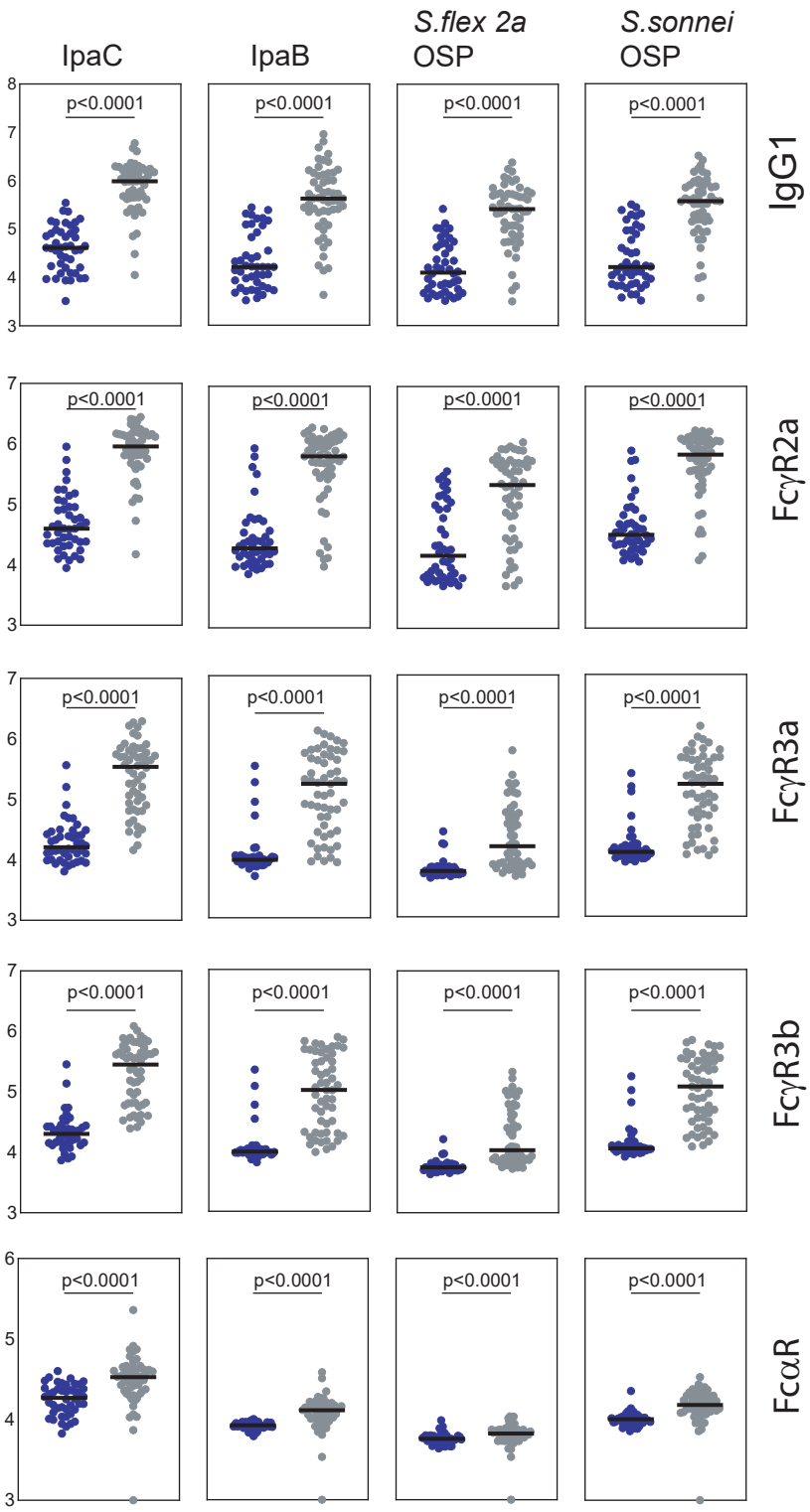
IpaB (WRAIR, Silver Spring, MD, USA), IpaC (WRAIR, Silver Spring, MD, USA), IpaD (WRAIR, Silver Spring, MD, USA), *S. flexneri* 2a 2457T LPS (WRAIR, Silver Spring, MD, USA), *S. flexneri* 3a strain J17B LPS (WRAIR, Silver Spring, MD, USA), *S. flexneri* 6 LPS (icddrb, Dhaka, Bangladesh), *S. sonnei* Moseley LPS (WRAIR, Silver Spring, MD, USA), *S. flexneri* 2a OSP, *S. flexneri* 3a OSP, and *S. sonnei* OSP were used to profile -specific humoral immune response. OSP was purified from *S. flexneri* 2a Sf2a260214_1 LPS (icddrb, Dhaka, Bangladesh), *S. flexneri* 3a Sf3a050214_5 LPS (icddrb, Dhaka, Bangladesh), and *S. sonnei* Moseley LPS by acid hydrolysis and size exclusion chromatography and conjugated to BSA [18]. Tetanus toxin and Ebola Glycoprotein (Mabtech Inc, Cincinnati, OH, USA) were used as a control. Protein antigens were coupled to magnetic Luminex beads (Luminex Corp, Austin, TX, USA) by carbodiimide-NHS ester-coupling (Thermo Fisher, Waltham, MA, USA). OSP and LPS antigens were modified by 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium and conjugated to Luminex Magplex carboxylated beads. Antigen-coupled microspheres were washed and incubated with plasma samples at an appropriate sample dilution (1:50 for Isotypes and 1:100 for all Fc- receptors) for 2 hours at 37°C in 384-well plates (Greiner Bio-One, Monroe, NC, USA). Unbound antibodies were washed away, and antigen-bound antibodies were detected by using a PE-coupled detection antibody for each subclass and isotype (IgG1, IgG2, IgG3, IgA1, IgA2 and IgM; Southern Biotech, Birmingham, AL, USA), and Fcγ-receptors were fluorescently labeled with PE before addition to immune complexes (FcγR2A, FcγR2B, FcγR3A, FcγR3B, FcαR; Duke Protein Production facility, Durham, NC, USA). After one hour incubation, plates were washed, and flow cytometry was performed with an IQue (Intellicyt, Albuquerque, NM, USA), and analysis was performed on IntelliCyt ForeCyt (v8.1).

Antibody-dependent monocyte and neutrophil phagocytosis (ADMP and ADNP)

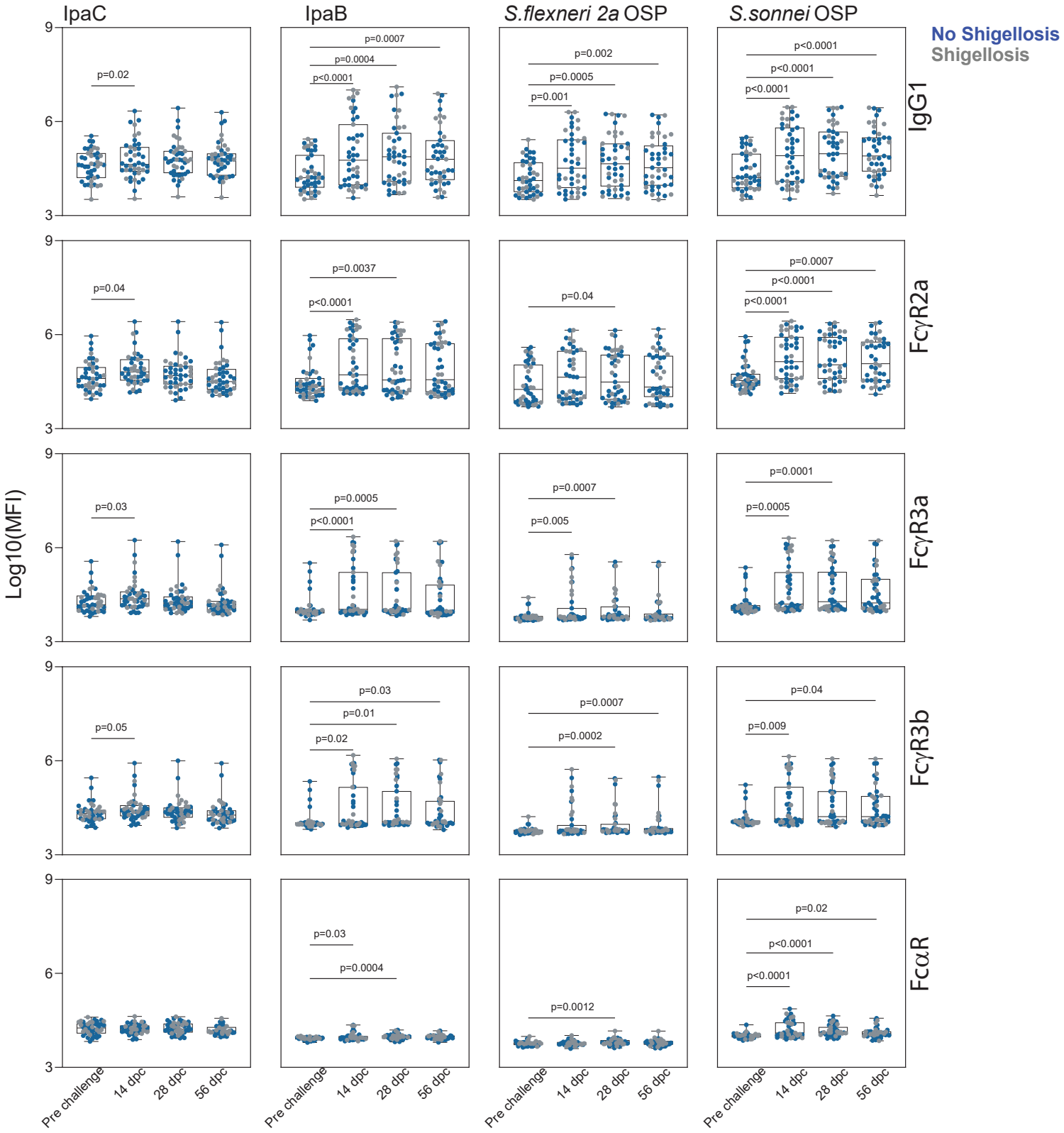
IpaB was biotinylated using Sulfo-NHS-LC-LC biotin (Thermo Fisher, Waltham, MA, USA) and coupled to fluorescent Neutravidin-conjugated beads (Thermo Fisher, Waltham, MA, USA). *S. flexneri* 2a OSP was modified with DMTMM and coupled to carboxylated fluorescent beads (Thermo Fisher, Waltham, MA, USA). To form immune complexes, a mix of both, or *S. flexneri* 2a OSP only antigen-coupled beads was incubated for 2 hours at 37°C with diluted samples (1:200) and then washed to remove unbound immunoglobulins. Primary human cells were isolated from blood of healthy donors collected by the Ragon Institute of MGH, MIT and Harvard. For ADMP, the immune complexes were incubated for 4 hours with fresh blood monocytes isolated with commercially available kit (StemCell Technologies, Cambridge, MA, USA) ($1.25x10^5$ monocytes/mL) and for ADNP for 1 hour with fresh blood neutrophils isolated from healthy donors with commercially available kit (StemCell Technologies, Cambridge, MA, USA). Following the incubation, cells were fixed with 4% PFA. For ADNP, neutrophils were washed, stained for CD66b (Biolegend, San Diego, CA, USA), and then fixed in 4% PFA. For ADMP, monocytes were washed, stained for CD14 (Biolegend, San Diego, CA, USA), and then fixed in 4% PFA.

Computational analysis

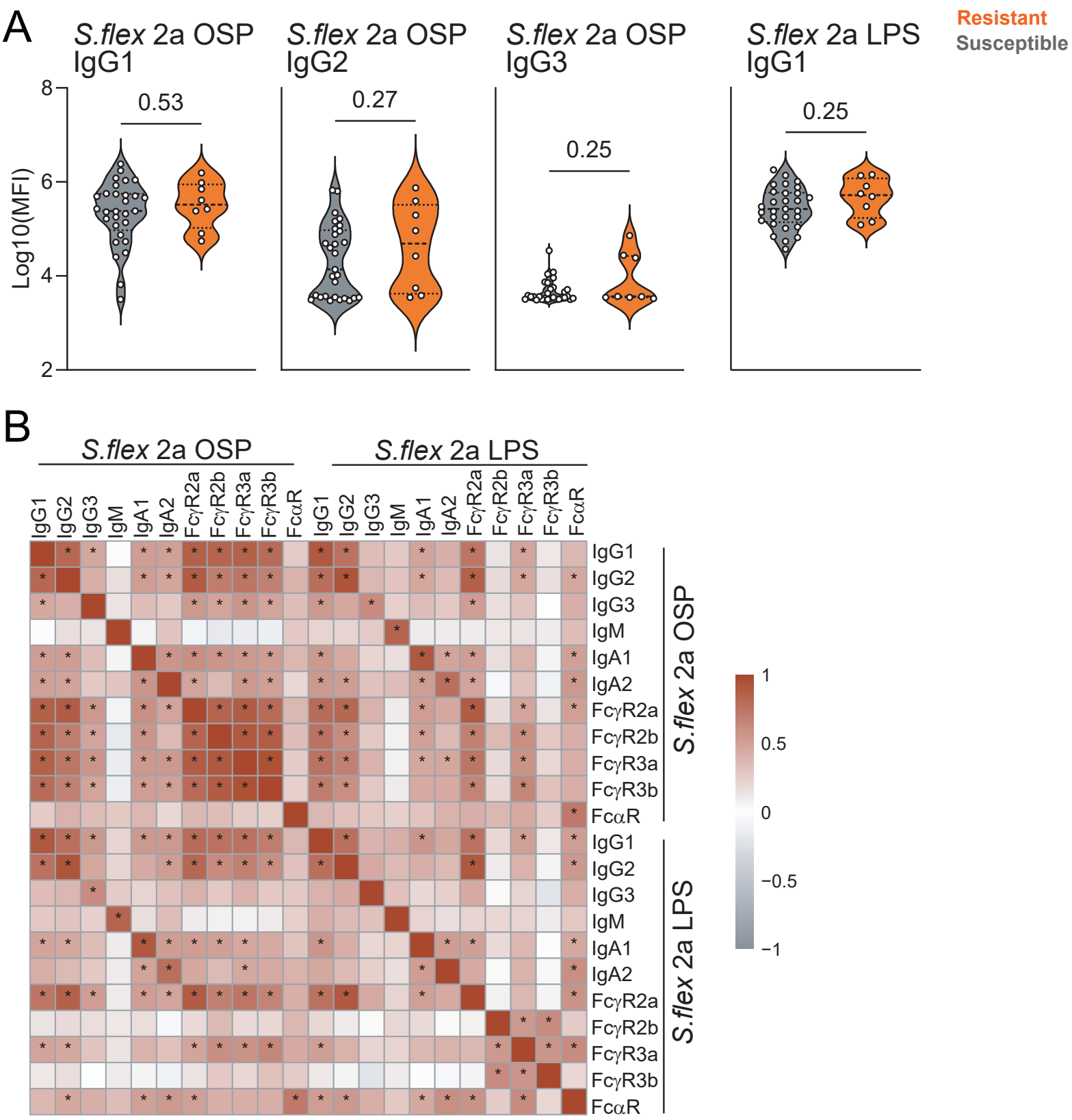
LASSO identified a minimal set of features that drives separation in samples of endemic or non-endemic exposure and varying disease severity. To estimate the minimal correlates that best explained group differences without overfitting, a five-fold nested validation framework was designed. In each repetition, the dataset was randomly divided into groups of 5 randomly assorted individuals, where 80% of the dataset was used for building the model and the remaining holdout set was used to test the model prediction, where the goodness-of-fitness of the model was measured by classification accuracy between groups. At each LASSO run lambda parameter was optimized using *cv.glmnet* function. Ultimately, this approach resulted in the generation of a model with the minimal set of features that generated the best classification prediction in a cross-validation test. LASSO selected features were used to build the PLS-DA model regressing against the disease severity score. The performance of the algorithm was evaluated with R^2 and Q^2 metrics. Features were ranked based on their Variable of Importance (VIP) score and the loadings of the latent variable 1 (LV1) was visualized in a bar graph, which captures the contribution of each feature to the variation in disease severity.



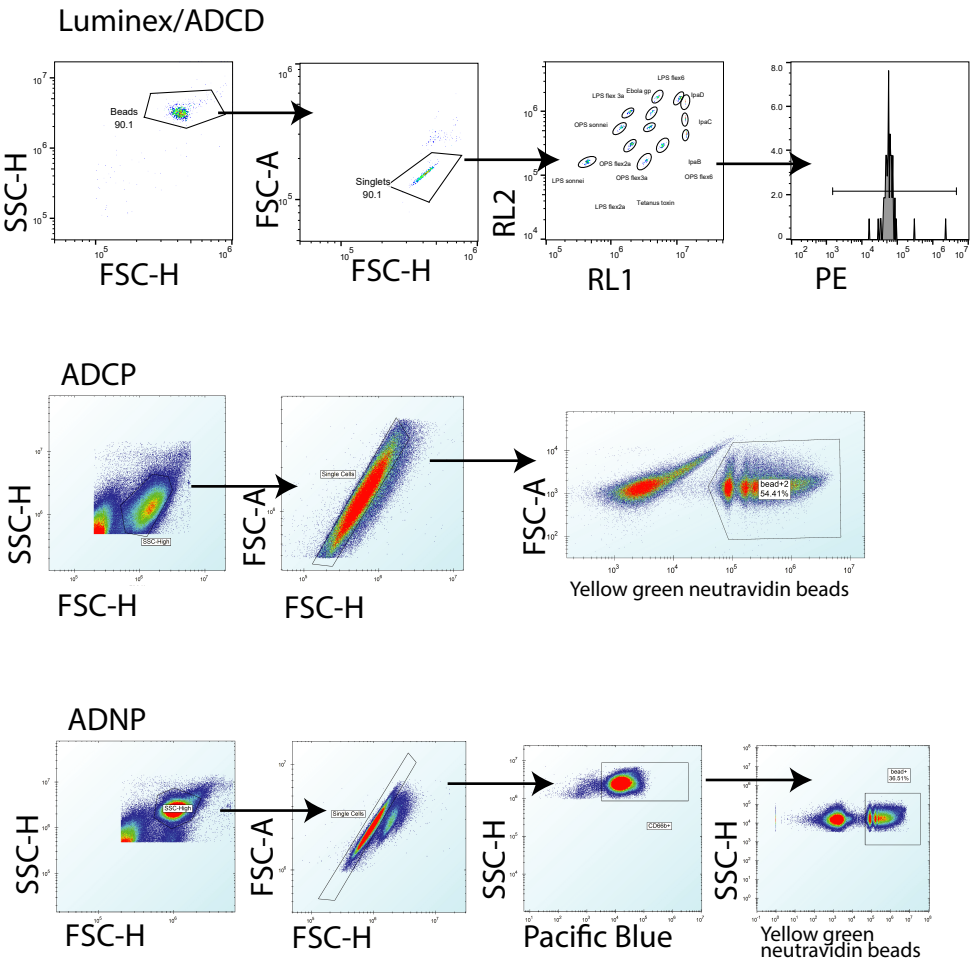
SuppFig1 -
Enrichment of FcR binding *Shigella* specific antibodies in individuals living in *Shigella* endemic region. Shigella-specific IgG1 and binding to Fcγr2a, Fcγr3a, Fcγr3b and FcαR in serum of individuals living in Shigella endemic or non-endemic regions. Horizontal line depicts median. (non-endemic n=44, endemic n=54) (p-value calculated by Mann-Whitney U test). t



SuppFig2 - Challenge induced antibody features.
Humoral evolution of IpaC, IpaB, S. flexneri 2a OSP and S. sonnei OSP-specific IgG1 and binding to Fcγr2a, Fcγr3a, Fcγr3b and FcαR in serum of individuals pre and post challenge with S. sonnei. (n=44)



SuppFig3 - IgG levels in shigellosis resistant and susceptible individuals
(A) S. flexneri 2a OSP-specific IgG1, IgG2 and IgG3 and S. flexneri 2a LPS-specific IgG1 levels in shigellosis resistant and susceptible individuals at entry to camp. (p-value calculated by Mann-whitney U test)
(B) Heat map of Spearman rank correlation between S. flexneri 2a OSP-specific and LPS-specific antibody features in Peruvian recruits at entry to camp. (n=37) *Benjamini-Hochberg corrected p<0.01



Supp Fig4 - Gating strategy of flow cytometry analysis