Early and Innate Host Reponses to Enteric Fever



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Abstract

Salmonella enterica serovar Typhi (*S.* Typhi) and *S.* Paratyphi A are human-restricted pathogens estimated to cause 20 million cases of enteric fever each year. Due to lack of an animal model which fully recapitulates human disease, and difficulty studying infection prior to clinical presentation in field settings, little is known about host-pathogen interactions during the incubation period. It is likely during this period of time that both vaccine-mediated and natural elimination of infection occurs in those who remain healthy despite exposure. Stool shedding, early plasma cytokine responses and *S.* Typhi DNAaemia have previously been detected in both volunteers who did and did not develop acute enteric fever following ingestion of *S.* Typhi Quailes strain. Here we have applied functional genomics and multiplex cytokine assays to further investigate the nature of the early host response and identify genetic risk factors for development of disease in human volunteers challenged with *S.* Typhi and *S.* Paratyphi A.

Perturbation of blood plasma cytokines was greatest 12 hours post-challenge and during acute disease. Early after challenge there was a significant rise in BDNF and EGF ($p < 0.0001$ compared with baseline, linear modelling), validated by independent assays and significant relative to unchallenged controls ($p < 0.005$, Mann–Whitney U test). In a multiplex cytokine assay produced by a different manufacturer, CD40L and GRO-α were also significantly raised after challenge ($p < 0.01$, Mann–Whitney U test). BDNF, EGF and GRO-α were increased in the supernatants of leukocytes stimulated with *S.* Typhi ($p < 0.1$, t-test). While GRO-α was secreted by *S.* Typhi infected granulocytes, monocytes, lymphocytes and intestinal organoids, EGF and CD40L were secreted by granulocytes only. IL1RA, MIG and IP10 were identified as correlates of symptomology and inflammation during acute disease ($p < 0.001$ for overall correlations with symptom score and C-reactive protein, Pearson correlation coefficient), and validated using data from two independent cohorts. *In vitro*, a greater amount of IL1RA was produced in response to *S.* Paratyphi A and *S.* Typhimurium than *S.* Typhi Quailes strain ($p = 0.03$ and 0.01 respectively, t-test), and appeared to be specifically secreted by granulocytes. No marked differences in plasma cytokine profile were seen between participants challenged with wild-type *S.* Typhi Quailes strain and an isogenic typhoid toxin knockout.

316 genes were significantly differentially expressed (FDR < 0.05, linear modelling) 12 hours post-challenge, normalised to gene expression changes in an unchallenged cohort. These changes were validated in two independent challenge studies, and correlated with gene expression changes following *in vitro* infection of cell lines ($\rho = 0.7$ and $p = 1 \times 10^{-48}$ for Hep2 cells, Pearson correlation coefficient) and inflammatory bowel disease ($\rho = 0.3$ and $p = 3 \times 10^{-5}$ with Crohn's disease, Pearson correlation coefficient). Gene sets relating to innate immunity were over-represented in the early host response, with deconvolution suggesting an early peak in neutrophils ($p = 1.6 \times 10^{-6}$ for the discovery cohort, Wilcoxon signed-rank test). *In vitro*, *S.* Typhi infection induced neutrophil activation, bacterial uptake and a rise in reactive oxygen species. Despite activation, bacterial survival was no different between granulocytes and monocytes four hours after *in vitro* infection ($p = 0.3$, t-test). No significant differences were observed between those who remained healthy and those who developed acute disease, although the magnitude of the early response was on average higher in those remaining healthy ($p = 3.7 \times 10^{-12}$, Mann–Whitney–Wilcoxon test).

At the genome-wide level, no polymorphisms were significantly associated with susceptibility or resistance to enteric fever. *C1QC* mRNA was significantly higher at baseline in those exposed to *S.* Paratyphi A who remained healthy after challenge ($p = 0.0004$, linear modelling), but no differences in C1q protein were seen between outcome groups ($p = 0.9$, Mann-Whitney-Wilcoxon test). The prevalence of HLA type HLA-B*27:05 was higher in those who developed

acute disease (p = 0.006, logistic regression), and *in vitro* resulted in greater bacterial burden 24 hours after *S.* Typhi infection (p = 0.05, t-test).

Overall, there appears to be a marked and reproducible early host response in both those who do and do not go on to develop enteric fever following exposure. Although underpowered to detect baseline differences between outcome groups, we identify several promising candidate factors such as HLA type and early transcriptional response.

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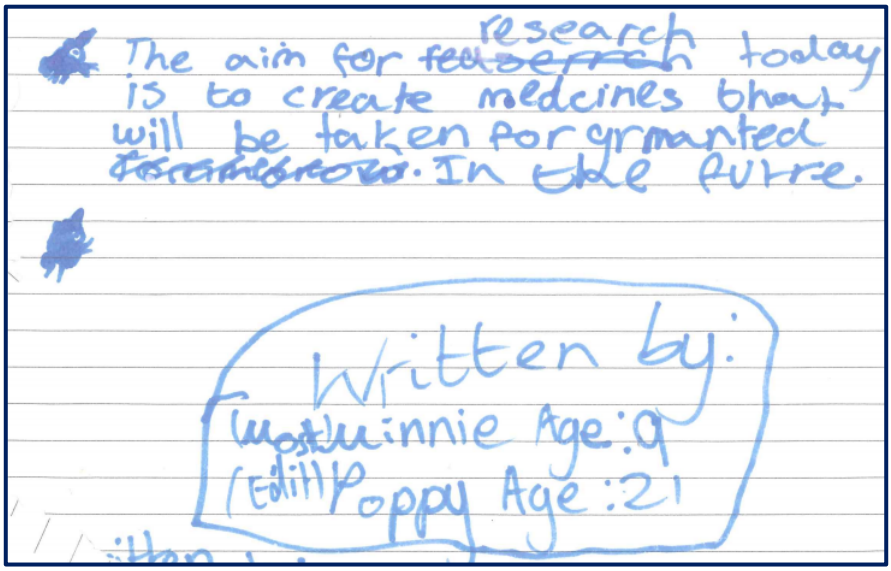
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Contributions

Running the human challenge studies		
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	Lead Doctor	Tom Darton
	Clinical team Leader	Tessa John
	Lead nurse	Kathryn Haworth
Typhoid oral vaccine trial	Chief Investigator	Andrew Pollard
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	Plasma processing	OVG Laboratory Staff
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	eBioscience multiplex cytokine assay data processing and analysis	Amber Barton
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	Merck Millipore multiplex cytokine assay (Typhoid toxin study and bicarbonate sub-study)	Amber Barton

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	Quality control and processing	Amber Barton
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	Optimisation of reactive oxygen species and bacterial uptake assay	Mari Johnson
	Optimisation of cell surface marker activation assay	Amber Barton
	Bacterial culture	Amber Barton and Elizabeth Jones
	Blood cell separation	Amber Barton
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	Flow cytometry	Amber Barton
	Tilt plating	Amber Barton and Jennifer Hill
	Merck Millipore multiplex cytokine assay (white blood cell supernatants)	Amber Barton
	Merck Millipore multiplex cytokine assay (organoids supernatants)	Amber Barton and Emily Lees

Abbreviations

ACK buffer	Ammonium-Chloride-Potassium buffer
APC	Allophycocyanin
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming units
CVD	Center for Vaccine Development
D0.5	Day 0.5 (12 hours post-challenge)
D10	Day 10 post-challenge
D14	Day 14 post-challenge
DC	Dendritic cell
E. *coli*	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eQTL	Expression quantitative trait loci
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G	Relative centrifugal force
GFP	Green fluorescent protein
GSEA	Gene set enrichment analysis
GWAS	Genome wide association study
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
iNTS	Invasive Non-typhoidal *Salmonella* Disease
LB	Lysogeny broth
LPS	Lipopolysaccharide
MAIT cell	Mucosal associated invariant T cell
MAPK	Mitogen-activated protein kinase
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Normalised Enrichment Score

Neu5Ac	N-Acetylneuraminic acid
Neu5Gc	N-Glycolylneuraminic acid
NK cell	Natural killer cell
NO	Nitric oxide
OVG	Oxford Vaccine Group
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP/Cy5.5	Peridinin Chlorophyll Protein Complex/Cyanine 5.5
qPCR	Quantitative polymerase chain reaction
ρ	Pearson correlation coefficient
RBC	Red blood cell
RIG-I-like receptors	Retinoic acid-inducible gene-I-like receptors
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
SCV	*Salmonella*-containing vacuole
S. Paratyphi A	*Salmonella enterica* serovar Paratyphi A
S. Typhi	*Salmonella enterica* serovar Typhi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
SED	Sub-epithelial dome
Sip	*Salmonella* Invasion Protein
SNP	Single nucleotide polymorphism
Sop	*Salmonella* Outer Protein
SPI	*Salmonella* Pathogenicity Island
Spv	*Salmonella* plasmid virulence
SSC	Side scatter
T_{CM}	Central memory T cells
TCR	T cell receptor
TD	Typhoid diagnosis
T_{EM}	Effector memory T cells
T_{EMRA}	CD45RA+ effector memory T cells
T_H1	Type 1 T helper cell
TLR	Toll like receptor
TN	Toxin-negative
TNF-R	Tumor necrosis factor receptor
T_{REG}	Regulatory T cell
T_{RM}	Tissue-resident memory T cells
TSH	Thyroid stimulating hormone
Vi capsule	Virulence capsule
Vi-PS	Vi capsular polysaccharide vaccine
Vi-TCV	Vi Polysaccharide-Tetanus Toxoid Conjugate Vaccine
WT	Wild-type

Genes

ADAM12	A disintegrin and metalloproteinase Metallopeptidase Domain 12
ADCY3	Adenylate Cyclase 3
ALKBH5	AlkB Homolog 5, RNA Demethylase
C1QBP	Complement C1q Binding Protein
C4BPA	Complement Component 4 Binding Protein Alpha
CAT	Catalase
CFB	Complement Factor B
CTSB	Cathepsin B
CTSK	Cathepsin K
CTSS	Cathepsin S
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCR3	C-X-C Motif Chemokine Receptor 3
DDIT4-AS1	DNA Damage Inducible Transcript 4 Antisense RNA 1
fepE	Ferric enterobactin transport protein
FKBP5	FK506 binding protein Prolyl Isomerase 5
GLDC	Glycine Dehydrogenase
IFI27	Interferon Alpha Inducible Protein 27.
IRF5	Interferon Regulatory Factor 5
ISG15	Interferon-stimulated gene 15
ITGB2	Integrin, Beta 2
KLF9	Kruppel Like Factor 9
KPNA1	Karyopherin Subunit Alpha 1
LY86	Lymphocyte Antigen 86
LYST	Lysosomal Trafficking Regulator
MAFF	MAF BZIP Transcription Factor F
MED23	Mediator Complex Subunit 23
MUC2	Mucin 2
MUC5B	Mucin 5B
NDUFB4	NADH:Ubiquinone Oxidoreductase Subunit B4
POT1-AS1	Protection Of Telomeres 1-Antisense 1
PRKCE	Protein Kinase C Epsilon
PRUNE2	Prune Homolog 2 With BCH Domain
PSMB8	Proteasome subunit beta 8
PTGR1	Prostaglandin reductase 1
PUM1	Pumilio RNA Binding Family Member 1
PYHIN1	Pyrin And HIN Domain Family Member 1
RALGDS	Ral Guanine Nucleotide Dissociation Stimulator
SKIV2L	Ski2 Like RNA Helicase
SLC37A2	Solute Carrier Family 37 Member 2
ST3GAL6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6
TGFA	Transforming growth factor alpha
TGFB	Transforming growth factor beta

TRAF3IP2	TRAF3 interacting protein 2
tviA	Vi polysaccharide biosynthesis protein TviA
UROS	Uroporphyrinogen III synthase
VAC14	VAC14 Component Of PIKFYVE Complex
vexB	Vi polysaccharide export inner-membrane protein
ZNF208	Zinc Finger Protein 208
ZSWIM1	Zinc Finger SWIM-Type Containing 1
EGF	Epidermal growth factor
HIF1A	Hypoxia-inducible factor 1-alpha
IL1RA	Interleukin 1 receptor antagonist
TGFA	Transforming growth factor alpha
TNFA	Tumor necrosis factor

Proteins

AHR	Aryl Hydrocarbon Receptor
BDNF	Brain-derived neurotrophic factor
BPI	Bactericidal/Permeability-Increasing Protein
C1q	Complement component 1q
C3b	Complement component 3b
C4b	Complement component 4b
CCL3	C-C Motif Chemokine Ligand 3
CD40L	Cluster of differentiation 40 Ligand
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
GRO-α	Protein product of CXCL1
hBD	Human beta-defensin
HD	Human defensin
HIF-1A	Hypoxia-inducible factor 1-alpha
IL1RA	Interleukin 1 receptor antagonist
IL1RB	Interleukin-1 receptor
IL2RG	Interleukin 2 Receptor Subunit Gamma
IP10	C-X-C motif chemokine 10
MCSF	Macrophage colony-stimulating factor
MIG	Monokine induced by gamma/CXCL9
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAI1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
PODXL	Podocalyxin-like protein 1
SDF1A	Stromal cell-derived factor 1
STAT3	Signal Transducer And Activator Of Transcription 3

TNFA	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGFD	Vascular Endothelial Growth Factor D

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Chapter 1 | Introduction

1.1 The burden of enteric fever

Enteric fever is caused by infection with the human-restricted bacteria *Salmonella enterica* serovars Typhi and Paratyphi A, B or C. Global typhoid burden estimates range from 11·9 million to 26.9 million cases per year (Antillón et al. 2017; Buckle, Walker, and Black 2012; Crump, Luby, and Mintz 2004; Crump and Mintz 2010; Mogasale et al. 2014), with an estimated case fatality rate of 2.5% (Pieters et al. 2018). Paratyphoid has an estimated incidence of 5.4 million cases per year, and may have a lower mortality rate (Crump and Mintz 2010). Enteric fever is primarily a childhood disease, peaking among 2-4 years of age in high burden settings and 5-14 years of age in lower burden settings (Antillón et al. 2017). While mortality is thought to be higher in younger compared with older children (Bhutta 1996), there is no significant difference in mortality between children and adults (Pieters et al. 2018).

Transmitted through faecal contamination of food and water, enteric fever is chiefly endemic in South Asia and Africa (Mogasale et al. 2014). In Kathmandu for example, 77% and 70% of drinking water samples contain detectable *S.* Typhi and *S.* Paratyphi A DNA respectively (Karkey et al. 2016). The percentage of enteric fever cases attributable to paratyphoid varies hugely by region, but is thought to account for a considerable proportion of enteric fever in Asia (Arndt et al. 2014). Although little is known about the relative incidence of *S.* Paratyphi A, B and C infection worldwide, *S.* Paratyphi A is currently the most common. At latitudes away from the equator, typhoid and paratyphoid follow the same seasonal pattern, coinciding with monsoon season in Asia, the dryer season in the Middle East, and irrigation season in Chile (Saad et al. 2018).

1.2 Status of enteric fever vaccines

The first vaccine against enteric fever, produced in 1896, was a whole-cell vaccine comprised of killed *S.* Typhi, *S.* Paratyphi A and *S.* Paratyphi B strains (Sztein, Salerno-Goncalves, and McArthur 2014). Although 65-88% efficacious, the vaccine was highly reactogenic, and its use was therefore discontinued (Vella 1972). At present there are two vaccines against *S.* Typhi approved by the FDA and member states of the European Medicines Agency (European Medicines Agency 2018a, 2018b): the Vi-polysaccharide vaccine and the live attenuated vaccine Ty21a. The Vi polysaccharide vaccine has an estimated efficacy of 69% for the first year after vaccination, and 55% for the first three years after vaccination(Anwar et al. 2014). However, as the Vi antigen generates a T-cell independent response this vaccine is not immunogenic in young children (Cadoz 1998). Covalent conjugation of polysaccharides to a carrier protein is thought to stimulate T cell help, resulting in B cell affinity maturation and class switching. Three such vaccines consisting of Vi conjugated to the tetanus toxoid have been licensed in India, of which Typbar-TCV has been pre-qualified by the World Health Organisation (Burki 2018). Vaccines using *Pseudomonas aeruginosa* exotoxin A and diphtheria toxoids as carrier proteins are also in the pipeline, as are vaccines using strong non-covalent interactions for conjugation (Sztein et al. 2014; Zhang, Lu, and Malley 2013).

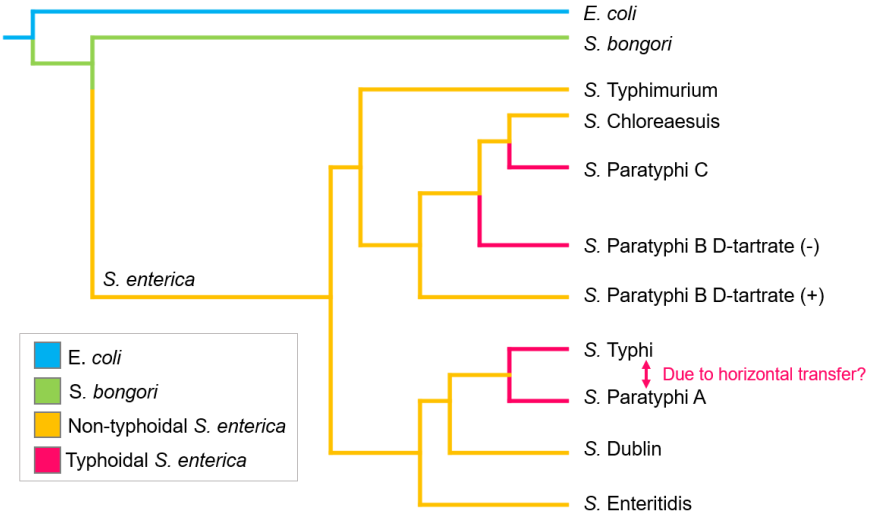
Ty21a, which is unable to produce galactose or the Vi capsule, was developed by chemical mutagenesis of the pathogenic *S.* Typhi strain Ty2 in the 1970s (Kopecko et al. 2009). Ty21a has an efficacy of 35-61% after three doses (Anwar et al. 2014). Given that compliance with three dose vaccine regimens is generally low, considerable effort has been put into engineering strains with the potential to confer protection after one dose. One such vaccine, M01ZH09, proved to be highly immunogenic but no more efficacious after one dose than the standard Ty21a regimen in a human challenge study (Darton et al. 2016). Other candidates, including Ty800, CVD 906, CVD 908, CVD 908-htrA, and CVD 909, induce robust humoral and cell-mediated immune responses, but efficacy is as yet unknown (Sztein et al. 2014).

No vaccines are currently licensed against paratyphoid. However several have undergone clinical immunogenicity trials, including a variety of LPS conjugates, and attenuated vaccine strain CVD 1902, which is deficient in guanine nucleotide synthesis and chaperone ATPase *clpX* (Martin et al. 2016; Wahid et al. 2019).

1.3 The evolution of typhoidal *Salmonella*

The genus *Salmonella* is thought to have diverged from *Escherichia coli* over 100 million years ago through the evolution of *Salmonella* pathogenicity island (SPI)-1, allowing it to invade non-phagocytic enterocytes in the intestine (Bäumler 1997). *Salmonella* is divided into two species: *S. bongori*, which primarily infects cold blooded hosts, and *S. enterica*, which through acquisition of SPI-2 is able to infect warm blooded animals and replicate within macrophages (Fookes et al. 2011). The species are further subdivided into serovars, defined by the antigenicity of their lipopolysaccharide (LPS) and flagella. Of these serovars, enteric fever is commonly caused by *S.* Typhi, *S.* Paratyphi A, and to a lesser extent by *S.* Paratyphi C and non-D-tartrate fermenting *S.* Paratyphi B (Figure 1.1). Human gastroenteritis and invasive non-typhoidal *Salmonella* (iNTS) disease is most commonly caused by *S.* Typhimurium and *S.* Enteritidis (Gal-Mor, Boyle, and Grassl 2014).

Figure 1.1: Simplified phylogenetic tree of *Salmonella enterica* and related species, based on PCR and microarray. Branches corresponding to typhoidal *Salmonella* serovars are highlighted in pink. Figure by A Barton, with reference to S. Porwollik et al. 2004; H. Kim, Park, and Kim 2006.



Broadly, typhoidal serovars are characterised by invasiveness, high genome degradation, ability to subvert the host response, and human restriction. Rather than arising from a common ancestor possessing these characteristics, these features appear to have been acquired by convergent evolution. Although *S.* Typhi is thought to have arisen approximately 50,000 years ago, there is very little diversity within this serovar, possibly as a consequence of a slow mutation rate (Gracioso, Hiruma-Lima, and Souza Brito 2002). At a whole-genome level, *S.* Paratyphi A is closely related to *S.* Typhi, leading to the suggestion that it arose from an *S.* Typhi-like strain acquiring serogroup A genes through horizontal gene transfer (McClelland et al. 2004). However it has more recently emerged that, whereas a quarter of their genomes are closely related, the remainder of the genome shows a much higher level of divergence (Didelot et al. 2007). This suggests that the two serovars are more likely to be distantly related *Salmonella* who underwent a burst of recombination in their human-restricted niche. *S.* Typhi and *S.* Paratyphi A have independently undergone genome degradation and developed separate mechanisms to evade host immunity (Hiyoshi et al. 2018). In contrast to *S.* Typhimurium, which has fewer than 40 pseudogenes, *S.* Typhi and *S.* Paratyphi A each possess over 150, including many of the genes required for intestinal colonisation. While this may have occurred after adaptation to a human-restricted niche, and represent degradation of genes unnecessary for

this lifestyle, in several cases pseudogenisation appears to have actively contributed to invasiveness (Crawford et al. 2013; Ortega et al. 2016; Valenzuela et al. 2015).

While non D-tartrate fermenting *S.* Paratyphi B can cause enteric fever, strains which do metabolise D-tartrate cause gastroenteritis (Chart 2003). Iterative clustering of 191 *S.* Paratyphi B genomes suggests that *S.* Paratyphi B is a genetically diverse serovar without a recent common ancestor, rather sharing a serovar classification due to horizontal transfer of flagellin genes (Pinna, Weill, and Peters 2016). However, within this set of genomes the invasive non-D-tartrate fermenting isolates were found to cluster together, their common ancestor predicted to date back to the 12th century. Although this cluster has lost several genes relative to the non-invasive clusters, including hydrogenases also absent in *S.* Typhi, the mechanism by which non-D-tartrate fermenting *S.* Paratyphi B causes invasive disease is unknown.

S. Paratyphi C is more closely related to swine pathogen *S.* choleraesuis than to other typhoidal serovars, diverging approximately 3000 years ago, and even having been recovered from an 800 year old skeleton (Zhou et al. 2018). As with the other typhoidal serovars, it appears to have undergone genome degradation independently (Liu et al. 2009). 85% of *S.* Paratyphi C isolates express the same immunomodulatory Vi capsule as *S.* Typhi, which may contribute to the ability of this serovar to cause systemic disease (Daniels et al. 1989).

While *S.* Typhimurium predominantly causes gastroenteritis in immune-competent humans, the variant ST313 is associated with invasive disease in sub-Saharan Africa (Kingsley et al. 2009). ST313 possesses 23 pseudogenes not found in other *S.* Typhimurium genomes, 11 of which are also absent from *S.* Typhi or *S.* Paratyphi A. Compared with the more common gastroenteritis-causing ST19 variant, ST313 is less cytotoxic, induces lower levels of inflammatory cytokines, and triggers less NF-κB activation (Ramachandran et al. 2015). Furthermore, its survival within macrophages is intermediate between non-typhoidal and typhoidal *Salmonella* serovars, as is its metabolic phenotype as measured by Raman spectroscopy (Xu et al. 2019). As in enteric fever

iNTS disease presents as fever and bacteraemia: however, it is a clinically distinct disease with higher mortality. Overall, ST313 may represent an intriguing evolutionary intermediate between non-typhoidal and typhoidal *Salmonella.*

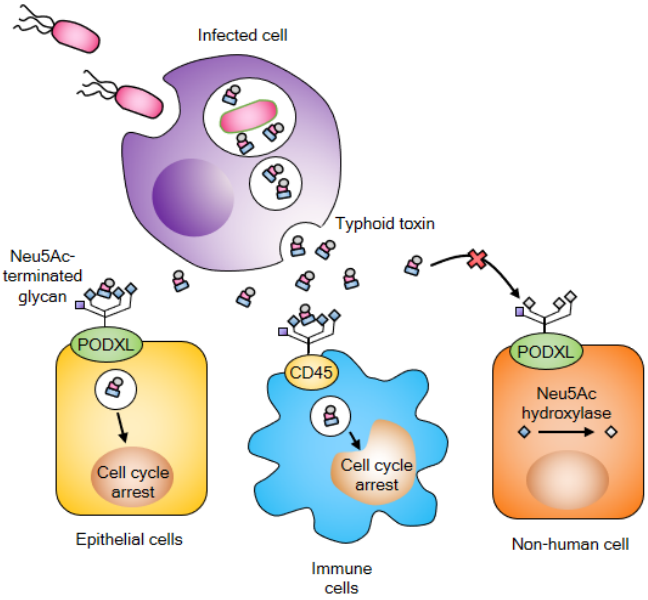
1.4 Host restriction

There are several proposed mechanisms to explain why *S.* Typhi is excluded from invasion of non-human hosts, in particular mice and livestock, while gastroenteritis-causing strains of *Salmonella* infect a variety of hosts.

1.4.1 Action of the typhoid toxin on human cells

In most non-human mammals, the sialic acid molecule which terminates the glycans on their cell surface, Neu5Ac, is hydroxylated to Neu5Gc. In humans however Neu5Ac hydroxylase has degraded into a pseudogene, and as a result human glycans are generally terminated by Neu5Ac. The typhoid toxin, secreted by intracellular *S.* Typhi and exported from the host cell (Figure 1.2), preferentially binds to Neu5Ac on the highly glycosylated cell surface proteins PODXL and CD45, suggesting it may target human cells only (Galán 2016). Wild-type mice, which possess both Neu5Gc and Neu5Ac terminated glycans, exhibit malaise and neutropenia when injected with the toxin (Song, Gao, and Galán 2013). Mice constitutively expressing Neu5Ac hydroxylase however are resistant (Gao et al. 2017). Nevertheless, knockout of the typhoid toxin does not affect invasion in humanised mice (Del Bel Belluz et al. 2016), competition with toxin-positive strains (Karlinsey et al. 2019), or the rate of acute disease in challenged human participants (Gibani, Jones, et al. 2019). Furthermore, the typhoid toxin is also present in a number of non-typhoidal *Salmonella* serovars (Rodriguez-Rivera et al. 2015). Thus it does not appear to play a role in systemic infection.

Figure 1.2: Secretion and action of the typhoid toxin. The typhoid toxin is secreted by intracellular *S.* Typhi and transported to the extracellular space. Here it binds to target cell receptors (PODXL on epithelial cells, CD45 on macrophages and T and B cells). The toxin preferentially binds to glycans terminated by Neu5Ac, characteristic of human cells. In the nucleus of target cells the DNase activity of the toxin causes DNA damage and cell cycle arrest. Figure by A. Barton, with reference to Galán 2016.



1.4.2 Adhesion to human cells using fimbriae

A convincing suggestion is that variation in fimbriae, extracellular protein structures which mediate adhesion to epithelial cells, determine host specificity. Recombinant *E. coli* expressing *S.* Typhi fimH are only able to strongly bind human epithelial cells, whereas *E. coli* expressing fimH from porcine, bovine and avian adapted *Salmonella* serovars specifically binds cells from their respective hosts (Yue et al. 2017). *S.* Typhimurium on the other hand weakly bound cells from a variety of hosts at equal affinity, possibly reflecting its niche as a non-invasive broad-specificity serovar.

1.4.3 Nutrient availability

Iron starvation of pathogens is an important mechanism of host defence, transferrin and lactoferrin acting as antimicrobial proteins through iron chelation (Gomme and McCann 2005; Kruzel, Zimecki, and Actor 2017). Infecting mice with *S.* Typhi in the presence of excess iron (resulting in saturation of host iron chelators) and 2,3-dihydroxy-benzoic acid (used by *S.* Typhimurium and presumably also *S.* Typhi to bind iron (Jones et al. 1977)), the LD_{50} was

decreased 150-750 fold (O'Brien 1982). In culture medium, *S.* Typhi is more sensitive to iron limitation than *S.* Typhimurium, which may explain why *S.* Typhimurium but not *S.* Typhi is capable of infecting mice (Karlinsey et al. 2019). However it is currently unknown whether iron starvation is less effective against *S.* Typhi in humans than in mice.

1.4.4 Host-specific toll-like receptors

Unlike humans, mice possess Toll-like receptor (TLR)11, which recognises flagellin and is highly expressed in the intestinal epithelium (Mathur et al. 2012). An initial report showed TLR11 knockout mice to be susceptible to *S.* Typhi infection, suggesting host restriction may occur through the action of host-specific TLRs. However, replication by the same and an independent group found the result to be unreproducible, which the original authors attribute to changes in the microbiome (Mathur et al. 2016; Song et al. 2016).

1.4.5 Rab32

S. Typhimurium produces the protease gtgE, allowing it to break down host protein Rab32, which is involved in the delivery of cargo to lysosome-related organelles (Spanò and Galán 2012). As a result, it is able to survive inside mouse macrophages, possibly by preventing delivery of antimicrobial proteins. *S.* Typhi however lacks gtgE, and is therefore killed. Although Rab32 deficient mice are more susceptible to *S.* Typhi (Spanò et al. 2016), as humans also possess Rab32 this can at most be a partial contributor to human host restriction.

1.5 Interaction with innate host defences

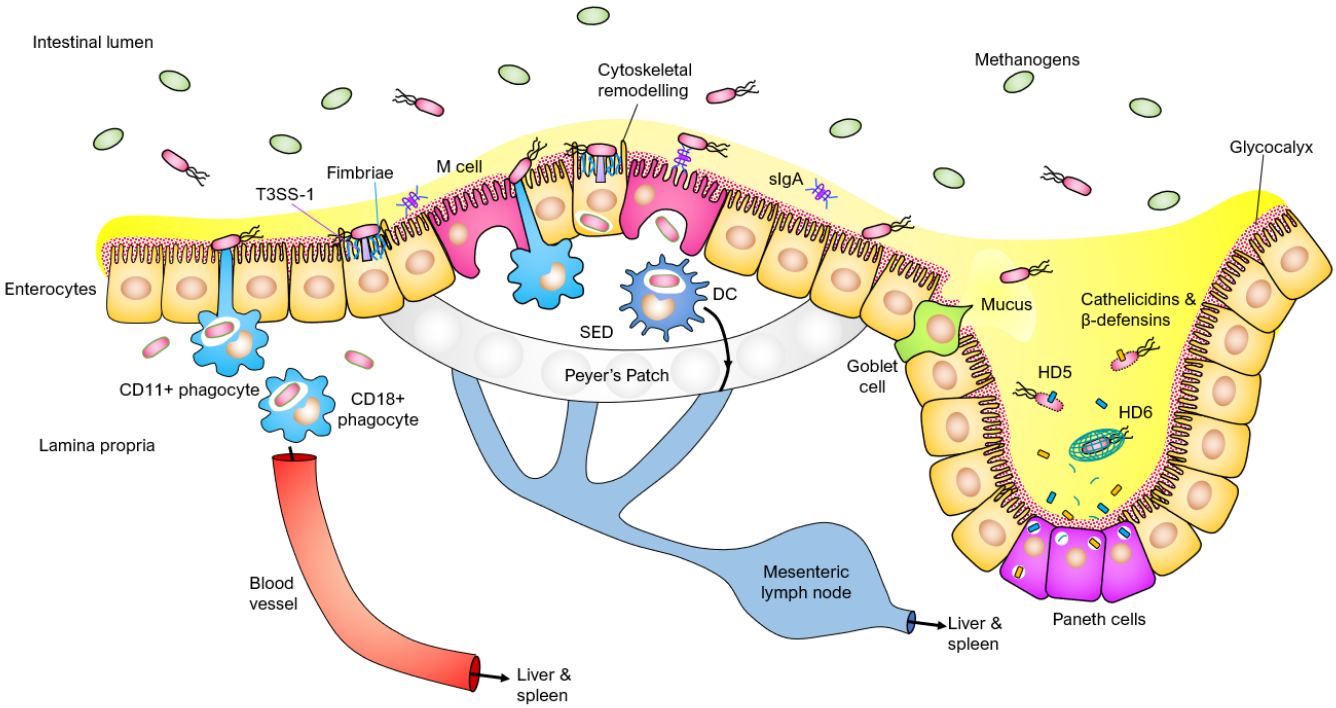
1.5.1 Anatomical barriers to infection

S. Typhi is transmitted through the ingestion of contaminated food and water, with a high estimated infectious dose; 10^3-10^4 organisms in naïve adults in a controlled human challenge model (Hornick et al. 1970; Waddington, Darton, Jones, et al. 2014). Following ingestion *S.* Typhi

faces hostile conditions in the stomach and small intestinal lumen before invading the terminal ileum, where its uptake by phagocytes allows systemic dissemination to the liver and spleen (Raffatellu et al. 2008) (Figure 1.3). The very first barrier to infection is gastric acid, low levels of which increases susceptibility to and severity of typhoid fever (Khosla, Jain, and Khosla 1993). While a high proportion of *S.* Typhi are likely killed in the stomach, for those which do survive, the exposure to acid may signal arrival in a new host, acting as a stimulus for replication (Ahirwar et al. 2014).

The human gut microbiota, consisting of commensal bacteria colonising the gut lumen and mucosa, is thought to play a key role in defence against pathogen invasion, and has previously been identified as associated with cholera susceptibility (Midani et al. 2018). The terminal ileum, where *S.* Typhi is thought to primarily invade, contains 10^7–10^8 bacteria per ml (Booijink et al. 2010). Unlike *S.* Typhimurium, which is able to metabolise butyrate produced by the microbiota and colonise the intestinal lumen, *S.* Typhi and *S.* Paratyphi A lack the required operon (Bronner et al. 2018). Despite this, a methanogen-rich faecal meta-transcriptome has been associated with a marginally lower risk of developing typhoid after human challenge, suggesting the microbiome may play a role in enteric fever susceptibility (Zhang et al. 2018).

Figure 1.3: Invasion of the intestinal epithelium by *S.* Typhi and systemic dissemination via phagocytes. *S.* Typhi invasion is impeded by mucus and the glycocalyx acting as physical barriers, as well as the action of defensins, cathelicidins and IgA in the intestinal mucosa. Those which successfully adhere to the epithelium cross by 1) T3SS1-mediated invasion of intestinal epithelial cells, or 2) uptake by CD11+ phagocytes sampling bacteria in the lumen. Bacteria in the lamina propria and sub-epithelial dome (SED) are then phagocytosed and systemically disseminated to the liver and spleen via the blood and lymph. Figure by A. Barton.



S. Typhi must then traverse the bactericidal layer of mucus coating the intestinal wall. *S.* Typhi infection induced upregulation of secreted gel-forming mucins *MUC2* and *MUC5B* in an organotypic intestinal model and *ex vivo* intestinal biopsies respectively (Nickerson et al. 2018; Salerno-Gonçalves et al. 2018). In addition to acting as a barrier, the mucus is enriched with antimicrobial peptides (Table 1.1) thought to disrupt bacterial cell membranes (Yang et al. 2004). Whereas Paneth cells in the small intestinal crypts secrete the two α-defensins HD5 and HD6, human β-defensins 1 and 2 (hBD1 and 2) and cathelicidin LL-37 are expressed by epithelial cells throughout the small and large intestine (O'Neil et al. 1999).

Table 1.1: Antimicrobial peptides present in the intestinal mucosa

Class	Peptide	Source
α-defensins	HD5	Paneth cells
	HD6	
β-defensins	hBD1	Intestinal epithelial cells
	hBD2	
Cathelicidins	LL-37	

The well-established role of α-defensins in fighting enteric infection has for the most part arisen from mouse studies, using related serovar *S.* Typhimurium as a surrogate for human-restricted *S.* Typhi. For example, a study using intestinal mouse organoids found mouse α-defensins to be capable of restricting growth of luminal *S.* Typhimurium (Wilson et al. 2015). Mouse α-defensin expression however is insufficient to prevent mortality when challenged with high dose *S.* Typhimurium, whereas transgenic expression of human HD5 in mice is fully protective (Salzman et al. 2003). As a possible means of immune evasion, orally administered *S.* Typhimurium reduces α-defensin expression in the Paneth cells of mice (Wahid et al. 2015). Although experiments on human small intestinal xenografts found *S.* Typhi to have no effect on α-defensin expression (O'Neil et al. 1999), HD5 expression is significantly lowered in small-intestinal biopsies from volunteers administered the Ty21a *S.* Typhi vaccine strain (Simuyandi and Kapulu 2016).

Whereas HD5 is directly antimicrobial, transgenic HD6 expression in mice does not affect survival of *S.* Typhimurium in the intestinal lumen (Chu et al. 2012). HD6 was, however, found to reduce both bacterial count in the Peyer's patches and mortality, attributed to its ability to form net-like structures around bacteria to prevent epithelial invasion. HD6 molecular nets have also been observed in intestinal aspirates from humans, and could thus potentially play a part in *S.* Typhi protection. The role of β-defensins is less well-defined; whereas hBD1 is constitutively expressed in the intestinal epithelium, hBD2 is upregulated in human intestinal xenografts infected with *S.* Typhi, likely as a result of NF-κB activation (O'Neil et al. 1999). Although recombinant β-defensins have been shown to be bactericidal against *S.* Typhi *in vitro*, and protective in mice infected intraperitoneally with *S.* Typhi (Maiti et al. 2014), neither experiment adequately recapitulates enteric infection. Likewise, although cathelicidin LL-37 shows some *in vitro* anti-*S.* Typhimurium activity, its importance *in vivo* is unknown (Turner et al. 1998).

In addition to cathelicidins and defensins, secretory IgA is thought to protect the intestinal epithelium by blocking bacterial uptake and inhibiting flagellar motility (Mantis, Rol, and Corthésy 2011). Given that serum IgG against *S.* Typhi was present in 29% of human challenge study participants at baseline (Darton et al. 2016), and that oral vaccination with *S.* Typhi strain Ty21a elicits a cross-reactive IgA response to non-typhoidal *Salmonellae* (Kantele et al. 2012); it is possible that even typhoid-naïve humans could express intestinal IgA capable of binding *S.* Typhi. Although a basal IgA response might prevent *S.* Typhi from invading the intestinal wall, the binding of IgA to M cells could enhance bacterial uptake, as has been observed with *Shigella* (Boullier et al. 2009). In support of this, mice lacking the polymeric immunoglobulin receptor required for IgA transport to the intestinal lumen are significantly more resistant to *S.* Typhimurium infection (Betz et al. 2018).

Underneath the mucous layer the glycocalyx, composed of glycolipids and glycoproteins extending from the plasma membrane of intestinal epithelial cells, serves as an additional protective barrier. *S.* Typhimurium uses two glycosyl hydrolases, nanH and malS, to degrade the glycocalyx, without which it is cannot efficiently invade (Arabyan et al. 2016). While *S.* Typhi does not possess nanH, malS is present with >90% sequence identity. *S.* Typhimurium infection also induces expression of host fucosyltransferases, resulting in highly fucosylated surface glycans which may enhance pathogen tolerance (Pickard et al. 2014).

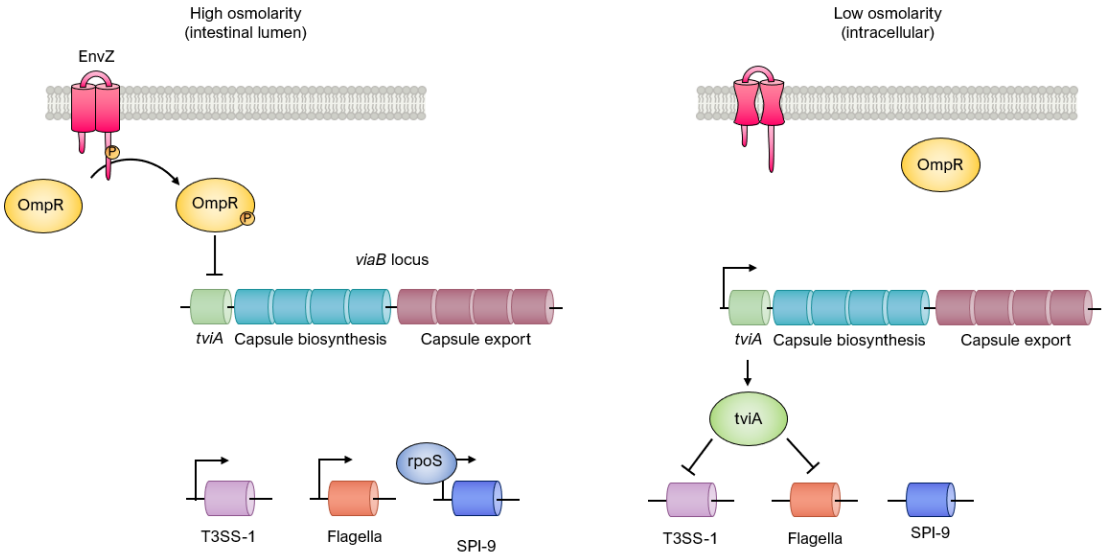
1.5.2 Invasion of the intestinal epithelium

At the surface of the intestinal epithelium, *S.* Typhi attaches to epithelial cells using fimbriae (Berrocal et al. 2015; Dufresne, Saulnier-Bellemare, and Daigle 2018; Leclerc et al. 2016). Sensing the high osmolarity of the intestinal lumen via the EnvZ/OmpR system, *S.* Typhi downregulates expression of TviA, a key regulatory protein encoded within the *viaB* operon in the serovar-specific SPI-7 locus (Nuccio, Rüssmann, and Bäumler 2010) (Figure 1.4). The drop in TviA in turn upregulates expression of flagellin and the Type III secretion system 1 (T3SS-1), allowing

invasion of the intestinal epithelium (Nuccio et al. 2010). High osmolarity also induces *S.* Typhi to express SPI-9, enhancing adherence to epithelial cells (Velásquez et al. 2016). Numerous mouse studies have suggested *S.* Typhimurium and *S.* Typhi preferentially target microfold cells, which capture and pass antigens from the intestinal lumen to the underlying Peyer's patches (Carlson and Jones 1998; Clark et al. 1994; Kohbata and Yokoyama 1986; Pascopella et al. 1995). However, *S.* Typhi lacks the long polar fimbriae used by *S.* Typhimurium to recognise M cells, showing preferential invasion of epithelial cells in a co-culture model, and no preference for M cells in a 3D human intestinal cell culture model (Gonzales, Wilde, and Roland 2017; Radtke et al. 2010).

The chief mechanism of epithelial invasion by all *Salmonella* serovars is mediated by T3SS-1, a molecular needle encoded by the relatively conserved SPI-1 locus (Sabbagh et al. 2010). *S.* Typhimurium injects effector proteins SipA, SipC, SopE, and SopE2 into the host cytosol, activating Rho-family GTPases and thus inducing cytoskeletal rearrangement so as to engulf the bacteria. In *S.* Typhi SopA and SopE2 are both pseudogenes (Ibarra and Steele-Mortimer 2009; Sabbagh et al. 2010; Valenzuela et al. 2015) Despite lacking these SPI-1 effectors, *S.* Typhi infection induces the enterocyte cytoskeleton to protrude at sites of invasion (Nickerson et al. 2018). In response to invasion enterocytes downregulate genes involved in cytoskeleton remodelling. This may represent a host response to prevent further infection, as pharmacological inhibition of actin or microtubule assembly almost entirely blocks *S.* Typhi uptake.

Figure 1.4: Genetic regulation in response to changes in osmolarity. In the high osmolarity intestinal lumen, EnvZ auto-phosphorylation activity is high, ultimately resulting in the phosphorylation of OmpR and suppression of the *viaB* locus. Thus, Vi capsule biosynthesis is supressed, while T3SS-1 and flagellin expression can take place. In the lower osmolarity environment inside cells, EnvZ undergoes a conformational change which reduces auto-phosphorylation. The *viaB* locus is hence expressed, resulting in synthesis of TviA and the Vi capsule. Regulatory protein TviA then goes on to supress T3SS1 and flagellin expression. Under high osmolarity conditions SPI-9 is also transcribed by a mechanism dependent on rpoS. Figure by A. Barton, with reference to Nuccio, Rüssmann, and Bäumler 2010; Velásquez et al. 2016; Santander, Roland, and Curtiss III 2008.



To avoid excessive Rho-family GTPase stimulation, which would lead to the activation of NF-κB (Winter et al. 2014), *S.* Typhimurium effector SptP reverses these changes to the cytoskeleton, while the effector AvrA blocks nuclear translocation of p65 (Collier-Hyams et al. 2002). In *S.* Typhi however a different strategy is required, as the gene encoding AvrA is absent and its translated SptP protein is too unstable to be secreted. Rather, sensing the drop in osmolarity as it enters the cell, *S.* Typhi upregulates TviA, resulting in concomitant downregulation of T3SS-1 (Nuccio et al. 2010). By doing so, excessive Rho-family GTPase stimulation is avoided, preventing the associated induction of NF-κB signalling (Winter et al. 2014). It is unclear which of these strategies is more successful: whereas *S.* Typhi induces greater NK-κB and MAPK responses than *S.* Typhimurium in infected Henle-407 cells, in *ex vivo* infected intestinal biopsies the opposite appears to be true (Hannemann and Galán 2017; Nickerson et al. 2018). Unlike *S.* Typhimurium, *S.* Typhi does not induce STAT3 activation

(Hannemann and Galán 2017), likely due to its lack of SopE2 (Ruan et al. 2017) and SarA (Jaslow et al. 2018).

Although T3SS-1 dependent invasion is well characterised, a number of proposed alternative entry mechanisms may in fact render this pathway redundant (Chowdhury et al. 2015, 2019; Radtke et al. 2010). For example, the requirement of $CD11^+$ phagocytes for an attenuated *S.* Typhimurium strain to cross a monolayer of intestinal cells has led to speculation that these cells facilitate uptake, sending trans-epithelial dendrites into the lumen to sample bacteria (Rescigno et al. 2001). Originally thought to be dendritic cells, these phagocytes have more recently been identified as tissue resident macrophages (Persson, Jaensson, and Agace 2010).

Having passed through the epithelium, *S.* Typhimurium flagellin activates TLR-5 at the basolateral surface, giving rise to neutrophil recruitment and inflammatory diarrhoea (Keestra-Gounder, Tsolis, and Bäumler 2015; Rhee et al. 2005). In *S.* Typhi however, the additional role of TviA in mediating downregulation of flagellin limits TLR-5 recognition, as well as activating biosynthesis of the protective Vi polysaccharide capsule. Infection of human colonic explants with wild type *S.* Typhi elicits lower levels of IL8 expression relative to a Δ*tviA S.* Typhi strain (Raffatellu et al. 2005); however it is contested to what extent this effect is due to presence of the Vi capsule or downregulation of flagellin. On the one hand, free Vi can inhibit IL8 secretion from intestinal cells in the presence of Vi^- *S.* Typhi (Sharma and Qadri 2004). On the other hand, if the Vi-capsule encoding genes in the *viaB* locus are knocked out, but TviA is retained, suppression of IL8 secretion still takes place (Winter et al. 2008).

Although *S.* Typhi is able to pass through the epithelium without causing inflammatory diarrhoea, there is evidence to suggest a host cytokine response at this point. In an organotypic model of the intestinal epithelium, IL1β, IL17A, TNF-α, IL6, CCL3 and IL8 were released in response to *S.* Typhi infection (Salerno-Gonçalves et al. 2018; Salerno-goncalves, Kayastha, and Fasano 2019). Although secretion of TNF-α, IL6 and CCL3 was reduced in the absence of

macrophages, of these three cytokines only TNF-α was directly produced by macrophages in response to stimulation, suggesting that macrophages may modulate cytokine secretion by other cells in the intestinal mucosa. Interestingly, *S.* Paratyphi B induced greater secretion of IL6 and TNF-α than *S.* Paratyphi A or *S.* Typhi, while *S.* Paratyphi A induced greater secretion of CCL3 (Salerno-goncalves et al. 2019). Despite possessing the *viaB* locus, *S.* Typhi still stimulated IL8 secretion from the fibroblasts, endothelial and epithelial cells in the model, as did *S.* Paratyphi A and B (Salerno-Gonçalves et al. 2018; Salerno-goncalves et al. 2019). This may represent either dampened down but still significant IL8 release, or suggest that the Vi capsule was poorly expressed under these culture conditions. Likewise, there was a trend towards apical IL8 secretion by intestinal biopsies in response to *S.* Typhi, as well as significant apical secretion of IL10, IL2 and IL4 (Nickerson et al. 2018). However, there was no significant cytokine release at the basolateral side, which could account for the lack of neutrophil infiltration in typhoid fever. Dose-dependent induction of plasma cytokines sCD40L, fractalkine, GROα, IL1RA, EGF and VEGF is observed in typhoid challenge participants within 12 hours of challenge, the time point at which intestinal invasion is likely to take place: however the origin of this signal is as yet unconfirmed (Blohmke et al. 2016).

1.5.3 Dissemination via mononuclear phagocytes

Once in the lamina propria and subepithelial domes underlying the intestinal epithelium, *S.* Typhimurium is then internalised by $CD11c^{+}$ cells (Hopkins et al. 2000; Persson et al. 2010; Petrovska et al. 2004) thought to represent either macrophages or dendritic cells. Although *S.* Typhi can be phagocytosed, uptake of *S.* Typhi and *S.* Paratyphi A is reduced in the absence of functional flagella (Elhadad et al. 2015; Schreiber et al. 2015) and fimbriae (Berrocal et al. 2015), suggesting that phagocytes can also be actively invaded. As *S.* Typhi enters these cells its protective Vi capsule has a variety of immunomodulatory effects. Vi masks LPS from recognition by TLR-4 on the cell surface, preventing subsequent release of inflammatory cytokines TNF-α, IL6 or IL8 (Hirose et al. 1997; Raffatellu et al. 2005; R. P. Wilson et al. 2008). It

reduces phagocytosis-mediated by BPI, an antimicrobial protein that binds LPS (Balakrishnan, Schnare, and Chakravortty 2016). The Vi polysaccharide also binds putative tumour suppressor protein prohibitin, through which it can inhibit TLR-5 responses in the presence of exogenous flagellin (Garg and Qadri 2010). However, in the presence of serum, and in particular serum-derived haemoglobin, prohibitin-binding is inhibited and Vi becomes a TLR-2 agonist, resulting in IL8, TNF-α, IL6, and IL12p40 secretion. Despite lacking Vi, the percentage of $IL8^{+}$ macrophages following stimulation with *S.* Paratyphi A and B is not any greater than after stimulation with *S.* Typhi, suggesting either that these serovars possess alternative mechanisms to suppress IL8 secretion, or that the Vi capsule was not expressed under the experimental conditions tested (Salerno-goncalves et al. 2019).

Having formed a *Salmonella*-containing vacuole (SCV) within the cells, different *Salmonella* serovars have individual mechanisms to avoid damage and death by host-derived reactive oxygen and nitrogen species. In the absence of TLR-4 mediated NF-κB activation following *S.* Typhi infection, the transcription of inducible nitric oxide synthase (iNOS) is reduced, limiting synthesis of bactericidal NO (Kleinert, Schwarz, and Forstermann 2003; R. P. Wilson et al. 2008). The acidic environment of the SCV induces expression of SPI-2, a locus associated with intracellular survival (Liew et al. 2019). By an SPI-2 dependent mechanism, *S.* Typhimurium evades free radical-mediated killing by preventing assembly of NADPH oxidase at the phagosome membrane (Gallois et al. 2001; Vazquez-Torres et al. 2000). SPI-2 however appears to be redundant in mediating intracellular survival of *S.* Typhi in macrophages, despite the high level of conservation in this genetic region between *S.* Typhi and *S.* Typhimurium (Forest et al. 2010; Sabbagh et al. 2010). The redundancy could in part be due to deterioration of epithelial invasion regulator *marT* into a pseudogene in *S.* Typhi, creating a new open reading frame which appears to encode a novel H_2O_2-protective protein (Ortega et al. 2016). Furthermore, a eukaryote-like serine/threonine kinase unique to *S.* Typhi is induced in response to H_2O_2, promoting survival within macrophages and contributing to virulence in infected mice (Theeya

et al. 2015). The *suf* operon, involved in producing Iron-Sulphur clusters under oxidative stress, also appears to contribute to *S.* Typhi survival within macrophages (M. Wang et al. 2015). Within the cells, *S.* Typhi induces a metabolic switch from carbohydrate to lipid consumption, although the significance of this is not yet understood (Xu et al. 2019). Despite being able to initially evade killing by macrophages, phagocytes are likely important for the eventual resolution of disease; mutations in NADPH oxidase prevent mice from clearing *S.* Typhimurium infection and reduce the bactericidal activity of macrophages (Y. Li et al. 2017).

Rather than being killed, internalised *S.* Typhimurium induces macrophage death; however, it is as yet unknown whether this acts to benefit the bacteria or the host. Three mechanisms outlined in Table 1.2 have so far been identified; a rapid necrosis-like death ("pyroptosis") in which bacterial effector protein SipB activates caspase-1 (Brennan and Cookson 2000; Hersh et al. 1999; Monack et al. 1996); an autophagy-mediated death in which SipB disrupts mitochondria (Hernandez et al. 2003), and a delayed pyroptotic death in which virulence plasmid protein SpvB destabilises the actin cytoskeleton (Browne, Lesnick, and Guiney 2002; Monack, Detweiler, and Falkow 2001; Santos, Tsolis, and Bäumler 2001; van der Velden et al. 2000). In addition to causing cell death, the activation of capase-1 results in the processing and secretion of inflammatory cytokines IL1β and IL18, potentially recruiting further phagocytes to uptake the released *S.* Typhimurium. Both knockout of sipB in *S.* Typhimurium (Chen et al. 2015) and knockout of caspase-1 in mice (Monack et al. 2000) reduces the ability of *S.* Typhimurium to cause invasive disease, suggesting pyroptosis to be a key factor in systemic infection (Monack et al. 2000).

Table 1.2: Mechanisms of *S.* Typhimurium-induced macrophage cell death						
Mechanism	Timescale	SPI involved	Caspase-1 dependent	Effector	Effector also present in *S.* Typhi?	References
Rapid pyroptosis	1-2 hours	SPI-1	Yes	SipB	Yes	(Brennan and Cookson 2000; Hersh et al. 1999; Monack et al. 1996)
Autophagy-mediated	3-5 hours	SPI-1	No	SipB	Yes	(Hernandez et al. 2003)
Delayed pyroptosis	12-18 hours	SPI-2	Yes	SpvB	No	(Browne et al. 2002; Monack et al. 2001; Santos et al. 2001; van der Velden et al. 2000)

While there is some evidence to suggest that *S.* Typhi is equally cytotoxic through both rapid and delayed pyroptosis (Chen, Kaniga, and Galán 1996; Monack et al. 2001), other studies point towards a lower tendency than *S.* Typhimurum to induce macrophage death (Schwan et al. 2000; Winter et al. 2015). *S.* Paratyphi A appears to be more cytotoxic towards macrophages than *S.* Typhi(Salerno-goncalves et al. 2019). In addition to direct activation by bacterial effectors, the recognition of flagellin by the NLRC4 inflammasome can also induce caspase-1 activation, suggesting that the induced cell death could in fact represent a protective host response (Winter et al. 2015). The temporal association between pyroptosis and *S.* Typhimurium clearance has led to the suggestion that bacterial release from dying macrophages leaves *S.* Typhimurium vulnerable to uptake and killing by neutrophils (Miao et al. 2010). Although *S.* Typhi flagellin is a particularly potent NLRC4 activator (Yang et al. 2014), the TviA-dependent downregulation of flagellin appears to reduce NLRC4 activation and pyroptosis, potentially acting as a means of immune evasion (Winter et al. 2015). Overall, it is unclear whether the death of infected macrophages acts to benefit the bacteria or the host.

Once intracellular, two mechanisms disseminate *S.* Typhimurium systemically to the spleen and liver; the migration of infected $CD18^{+}$ phagocytes into the blood via the hepatic portal vein (Vazquez-Torres et al. 1999; Worley et al. 2006), and the putative trafficking of dendritic cells into the lymph nodes (McSorley et al. 2002; Sundquist and Wick 2005; Miguel A. Tam et al.

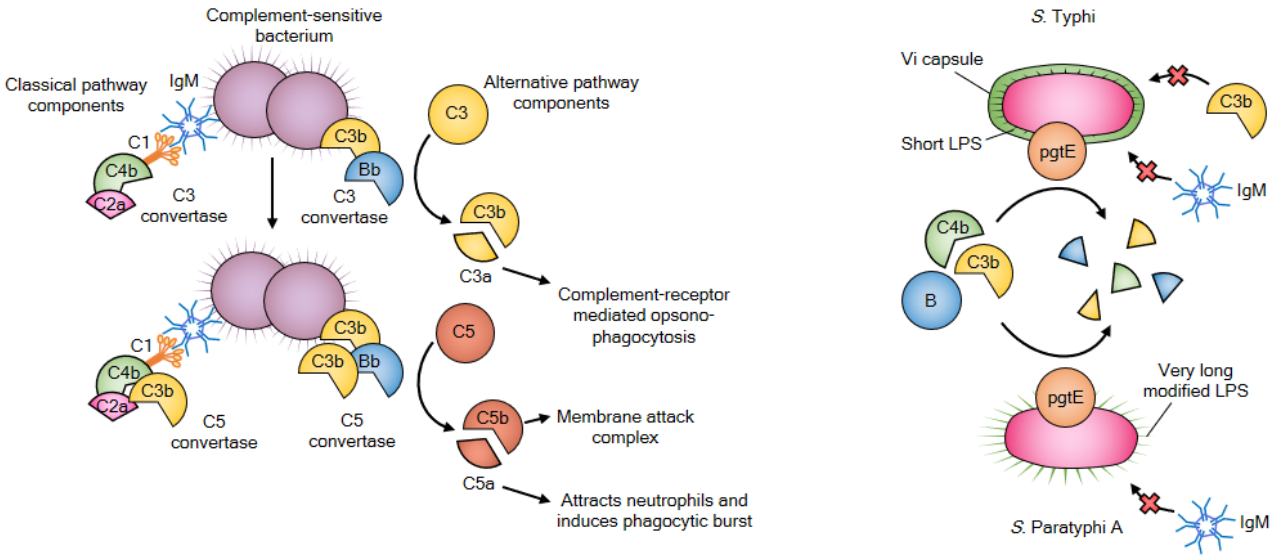
2008). The former mechanism is the more rapid, with primary bacteraemia occurring on a timescale of minutes (Gerichter 1960; Vazquez-Torres et al. 1999; Worley et al. 2006). The dissemination of *S.* Typhimurium via the lymph on the other hand takes place on a timescale of hours (McSorley et al. 2002; Miguel A. Tam et al. 2008). While live *S.* Typhi has not directly been isolated from the lymph or blood in the hours following human infection, *S.* Typhi DNA has been detected in the blood in the first 24 hours after challenge, and the mechanism of dissemination is presumed to be similar (Darton et al. 2017). After an incubation period of 7-14 days, during which the bacteria replicate within monocytes/macrophages in the spleen and liver, *S.* Typhi is once more released into the bloodstream in a secondary bacteraemia, resulting in the onset of clinical disease (Parry et al. 2002). In a study of clinical samples the majority of *S.* Typhi bacteria in the blood and bone marrow were reported to be within monocytes, while approximately one third were found to be extracellular (Wain et al. 1998, 2001). In many cases of Gram-negative bacteraemia, the recognition of LPS by TLR-4 and resultant release of TNF-α would give rise to life-threatening septic shock (Waage et al. 1989; Wortel et al. 1992). Although levels of TNF-α and IL6 are somewhat raised in typhoid fever, it seems the attenuation of TLR-4 signalling through the Vi capsule prevents inflammation reaching a fatal level (R. P. Wilson et al. 2008).

During acute disease the level of iron-regulating hormone hepcidin is significantly raised in the serum, resulting in the sequestration of iron within macrophages (Darton et al. 2015). While this response might restrict the growth of free bacteria through iron starvation, the diversion of iron to macrophages could be advantageous to bacteria residing intracellularly. As well as being required for bacterial growth, iron activates *S.* Typhi ferric uptake regulator (Fur), repressing sRNAs RfrA and RfrB, and enhancing H_2O_2 resistance and intracellular survival through an unknown mechanism (Leclerc, Dozois, and Daigle 2013).

1.5.4 Evading complement-dependent neutrophil activation

Rather than leaving extracellular bacteria vulnerable to complement, *S.* Typhi appears to have evolved several mechanisms to evade complement-dependent opsonisation and killing (Figure 1.5). In common with *S.* Typhimurium, *S.* Typhi possesses an operon which glucosylates the O-antigen on LPS to reduce C3 binding, as well as the conserved surface protease PgtE which cleaves C3b, C4b and B (Ramu et al. 2007; Riva, Korhonen, and Meri 2015; Uniprot n.d., n.d.). The lack of free hydroxyl groups in the Vi capsule produced by *S.* Typhi also prevents C3b from binding the bacterial surface (Wilson et al. 2011). This protection is enhanced by a nonsense mutation in the *fepE* gene, preventing synthesis of very long O-antigen chains which would expose hydroxyl groups to C3b at the capsule surface (Crawford et al. 2013). *S.* Paratyphi A not only lacks the Vi capsule, but also produces functional *fepE.* However, the O-antigen of *S.* Paratyphi A differs due to the pseudogenisation of *rfbE*, giving it a branching paratose residue which prevents IgM-mediated activation of the classical complement pathway (Hiyoshi et al. 2018).

Figure 1.5: Complement evasion by *S.* Typhi and *S.* Paratyphi A. The alternative and classical complement pathways culminate in the formation of C3 and C5 convertases, resulting in the attraction of neutrophils by C5a and opsonisation by C3b. In *S.* Typhi expression of the Vi capsule and absence of very long O-antigen chains prevents C3b and IgM deposition, while in *S.* Paratyphi A the production of very long modified O-antigens prevents IgM binding shorter O-antigen chains on its surface. Furthermore, the surface protease PgtE cleaves C3b, C4b and B. Figure by A. Barton.



Despite correlating with attenuated disease, serum bactericidal antibody has not yet been found to associate with protection in the human challenge model (Juel et al. 2018). This raises the possibility that the direct role of complement in S. Typhi lysis is secondary to its role in attracting and activating phagocytes. Inhibition of complement deposition by factors such as the Vi capsule reduces generation of chemoattractant C5a, impairing neutrophil recruitment (Wangdi et al. 2014). Although neutrophil blood counts drop in acute disease, the peripheral blood transcriptome is dominated by clusters associated with neutrophils, suggesting a considerable involvement in the immune response (Blohmke et al. 2016; Thompson et al. 2009; Waddington, Darton, Jones, et al. 2014). Calprotectin, a chelating protein complex which constitutes 40% of neutrophil cytosol, is raised in both the plasma and faeces of patients with typhoid fever, and is able to inhibit the growth of *S.* Typhi *in vitro* (De Jong et al. 2015). However, while *S.* Typhimurium infection induces upregulation of intestinal calprotectin in mice, calprotectin knockout mice are no more susceptible to infection. This is thought to be due to expression of a high affinity zinc transporter that can overcome calprotectin zinc chelation (Liu et al. 2013), and which is conserved between *S.* Typhi and *S.* Typhimurium. Therefore, the observed rise in calprotectin may not be protective against typhoid fever *in vivo.* As in macrophages, neutrophil phagocytosis of *S.* Typhi or *S.* Paratyphi A does not appear to stimulate a bactericidal oxidative burst by NADPH oxidase (Hiyoshi et al. 2018; Kossack et al. 1981; Miller, Garbus, and Hornick 1972; Roger et al. 1976). However, neutrophils have still been reported to kill 96% of *S.* Typhi *in vitro* within an hour (Kossack et al. 1981), the ability to do so in patients with chronic granulomatous disease suggesting that other killing mechanisms may be important (Looney and Steigbigel 1986). To an extent contradicting these studies which suggest neutrophils are highly bactericidal towards *S.* Typhi, in the early stages of mouse *S.* Typhimurium infection viable bacteria were predominantly recovered from neutrophils in the spleen, suggesting successful survival *in vivo* (Dunlap et al. 1992).

1.5.5 Natural killer cell stimulation

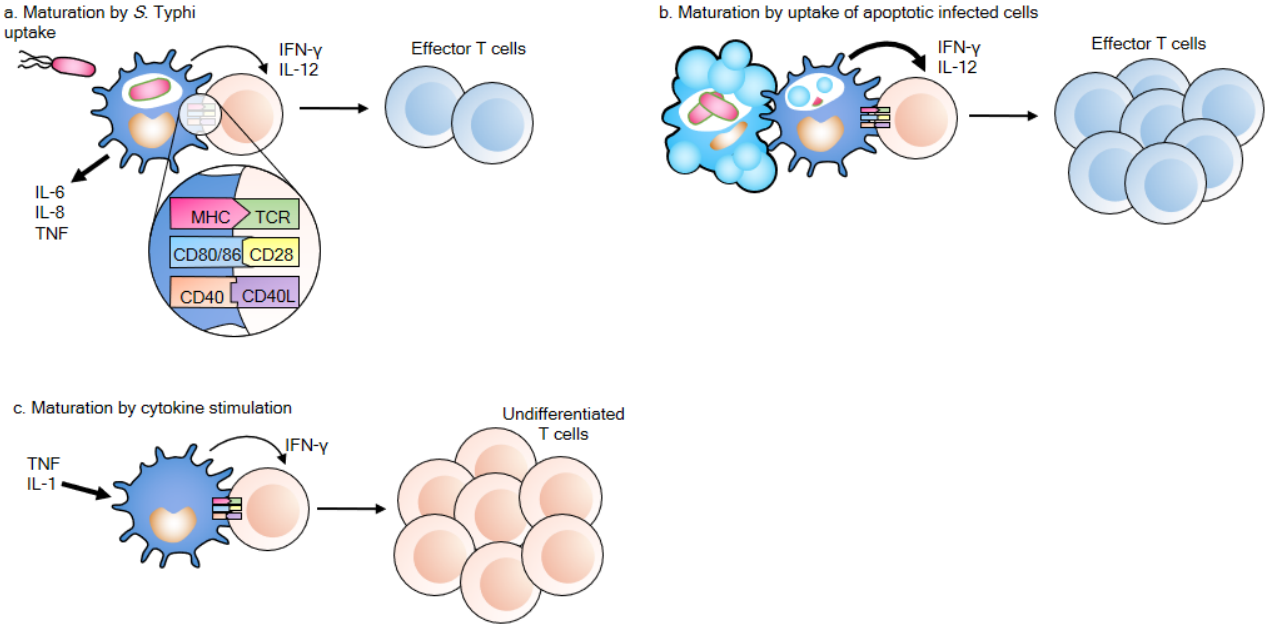
While the role of natural killer (NK) cells is well established in viral and cancer immunity, an emerging contribution to bacterial immunity has come to light. In mice infected with *S.* Typhimurium, IL18-mediated recruitment of NK cells to the gut did not affect bacterial load, but did increase intestinal inflammation (Müller et al. 2016). Although gastrointestinal inflammation is not a hallmark of human enteric fever as it is in mice, IL18 does rise in the plasma in acute disease (unpublished data- see 3.3.5), as does the percentage of NK cells producing granzyme A (de Jong et al. 2017). Furthermore, in participants receiving attenuated oral vaccine *S.* Typhi strain Ty21a, gene sets relating to NK cells were enriched in the whole blood transcriptome (Blohmke et al. 2017). *In vitro* stimulation of NK cells with fixed *S.* Typhi enhanced expression of activation marker CD69 as well as their killing ability, while stimulation with attenuated vaccine strains Ty21a and M01ZH09 increased the proportion of CD107a and IFN-γ-positive NK cells (Blohmke et al. 2017; Puente et al. 2000). However, it is yet to be determined whether NK cells play a role in enteric fever immunity *in vivo.*

1.5.6 Activation of the adaptive immune response by dendritic cells

In mouse models of typhoid, dendritic cells (DCs) are rapidly recruited to the Peyer's patches within 12 hours of oral *S.* Typhimurium infection (Salazar-Gonzalez et al. 2006; Shreedhar, Kelsall, and Neutra 2003). To activate the differentiation of naïve T cells, DCs must first undergo maturation, involving upregulation of MHC class II molecules and co-stimulatory molecules CD80, CD86 and CD40. Exposure of DCs to *S.* Typhi *in vitro* gives rise to dose-dependent upregulation of CD80, while human challenge with *S.* Typhi results in upregulation of CD40 (Salerno-Goncalves and Sztein 2009; Toapanta et al. 2015). The DCs also show increased expression of cell activation marker CD38, mature DC marker CD83 and complement receptor 2, suggesting a key role in the immune response to typhoid(Salerno-Goncalves and Sztein 2009; Toapanta et al. 2015).

DC maturation is instigated by three mechanisms (Figure 1.6); through the direct uptake of *S.* Typhi, through uptake of apoptotic *S.* Typhi-infected cells, and through stimulation by inflammatory cytokines. The first of these mechanisms represents the prototypical uptake of bacteria and presentation of peptides to $CD4^+$ and $CD8^+$ T cells. Of the three major DC subsets present in the lymphoid tissue ($CD8\alpha^+$, $CD4^+$, and $CD8\alpha^-CD4^-$), all are capable of directly phagocytosing *S.* Typhimurium (Yrlid and Wick 2002). In typhoid challenge participants, a peak in *S.* Typhi bound to the surface of DCs was apparent after 96 hours in those who did develop typhoid fever, but immediately after challenge in those who did not, suggesting that rapid DC uptake may play a role in preventing clinically evident typhoid from developing (Toapanta et al. 2015). Given the ability of *S.* Typhi TLR agonists OmpS1 and OmpS2 to induce upregulation of CD40 and MHC II in DCs (Moreno-Eutimio et al. 2013), and the reduced ability of *S.* Typhimurium-infected $MyD88^{-/-}$ DCs to activate T cells (Miguel A Tam, Sundquist, and Wick 2008), TLR signalling is thought to play a considerable role in direct maturation. By downregulating flagellin expression in a TviA-dependent manner however, *S.* Typhi evades both TLR recognition and the presentation of flagellin-derived peptides to T cells, preventing a flagellin-specific T cell response (Atif et al. 2014). Although DCs matured in this way produce inflammatory cytokines IL6, IL8 and TNF-α, only low levels of IL12 and IFN-γ, the cytokines responsible for inducing T_H1 differentiation, are produced (Salerno-Goncalves and Sztein 2009).

Figure 1.6: Mechanisms of dendritic cell activation in *Salmonella* infection a) Direct infection of dendritic cells results in secretion of IL12 and IFN-γ, stimulating T_H1 responses. However the uptake of apoptotic cells by dendritic cells (b) results in greater IL12 and IFN-γ secretion and is more effective at stimulating T cell expansion. Dendritic cell maturation can also be induced by cytokines (c), which induces T cell expansion but not T_H1 differentiation. Figure by A. Barton.



The alternative mechanism, in which DCs phagocytose apoptotic *S.* Typhi infected cells, does however induce high levels of IL12 and IFN-γ secretion (Salerno-Goncalves and Sztein 2009). It is proposed that *S.* Typhi LPS may reach the plasma membrane of infected cells, as has been observed in *Chlamydia* infection (Karimi, Schloemer, and Wilde 1989), activating TLRs on the surface of DCs as they take up the apoptotic cell fragments. *In vitro* infection of PBMCs from healthy volunteers suggests this mechanism to be equally if not more important than direct uptake for inducing DC cytokine production (Salerno-Goncalves and Sztein 2009). Furthermore, this manner of priming appears to be more effective in promoting the expansion of *S.* Typhi-specific T cells. Finally, cytokine stimulation can give rise to a DC surface marker phenotype characteristic of maturity, resulting in the upregulation of CD40, CD86 and MHC class II (Sporri and Reis e Sousa 2005). Although these DCs can induce T cell expansion, they do not secrete IL12, and are thus unable to induce T_H1 differentiation.

1.6 Interaction with adaptive host defences

1.6.1 Cell-mediated immunity

The cell-mediated and humoral responses both play important and complementary roles in immunity to *S.* Typhi; the cell-mediated response in targeting intracellular bacteria, and the humoral response in targeting extracellular bacteria (Sztein et al. 2014). Whether the cell-mediated or humoral response dominates varies between individuals, with little correlation in the size of the two responses (Dham and Thompson 1982; Salerno-Gonçalves et al. 2003; Tacket et al. 2000). Cellular immunity to *S.* Typhi is characterised by a strong IFN-γ response (Blohmke et al. 2016; Butler et al. 1993). Following stimulation by soluble *S.* Typhi antigens, $CD4^+$ lymphocytes are the predominant IFN-γ secreting cells amongst PBMCs from typhoid patients and CVD 908-*htrA* vaccinees (Bhuiyan et al. 2014; Salerno-Gonçalves et al. 2003; Sheikh et al. 2011). However, in PBMCs from Ty21a vaccinees stimulated instead by co-culture with *S.* Typhi infected cells, most IFN-γ secretion can be attributed to $CD8^+$ T lymphocytes (Lundin, Johansson, and Svennerholm 2002; Salerno-Goncalves, Pasetti, and Sztein 2002). Interestingly, the Vi polysaccharide inhibits anti-CD3 mediated T cell activation in mice, suppressing secretion of IFN-γ and IL2 (Santhanam et al. 2014).

The relative importance of $CD4^+$ and $CD8^+$ cells in resolving *S.* Typhi infection is unknown. Whereas the genetic association between typhoid susceptibility and polymorphisms in MHC Class II molecules would suggest that $CD4^+$ cells play an important role (Dunstan, Stephens, et al. 2001), the lack of a clear link between HIV infection and typhoid susceptibility implies otherwise (Crump et al. 2011; Gotuzzo, Frisancho, and Sanchez 1991; Keddy et al. 2016; Khan, Coovadia, and Sturm 1997). Nevertheless, there is a rise in proliferating ($Ki67^+$) $CD4^+$ T cells following *S.* Paratyphi A challenge, in particular a $CD38^+CCR7^-$ T cell subset which possesses mucosal homing markers and expresses MIP1-β and IFN-γ (Napolitani et al. 2018). This subset is enriched in *Salmonella*-specific T cells, primarily specific to antigens HlyE, CdtB, and PhoN. $CD4^+$ cells secrete IFN-γ in response to *S.* Typhi antigens, providing further evidence of a

primarily T_H1-polarised response (Bhuiyan et al. 2014). In addition to its well-established role enhancing microbial killing by macrophages (Spellberg and Edwards 2001), IFN-γ can induce macrophages to express tryptophan catabolising enzyme indoleamine 2,3-dioxygenase, potentially restricting *S.* Typhi growth through tryptophan starvation (Blohmke et al. 2016).

While the T_H1 response appears to predominate in acute typhoid, other $CD4^+$ subsets are also implicated. Antigen-specific T_H17 cells for example, thought to recruit neutrophils in the anti-bacterial immune response, are detectable during convalescence (Bhuiyan et al. 2014). Moreover, oral Ty21a vaccination increases levels of antigen-specific $CD107a^+$ $CD4^+$ cells with potential MHC class II-mediated cytotoxic activity (Wahid et al. 2016). The secretion of IL10 by *S.* Typhi antigen-stimulated PBMCs from Ty21a and CVD 909 vaccinated participants has led to speculation that anti-inflammatory T_{REG} cells are induced by attenuated *S.* Typhi strains (Wahid et al. 2007; Wyant, Tanner, and Sztein 1999). Furthermore, during acute disease there is a rise in serum kynurenine, a metabolite which induces T_{REG} differentiation (Blohmke et al. 2016). Although circulating T_{REG} numbers at baseline do not affect the outcome of typhoid challenge, upregulation of gut-homing molecule $\alpha_4\beta_7$ by stimulated T_{REG} cells has been associated with greater susceptibility to typhoid fever (McArthur et al. 2015). Thus, T_{REG} cells may home to the gut and suppress protective T_H1 and cytotoxic T cell responses.

It is currently unclear whether baseline $CD8^+$ are associated with the outcome of infection: whereas in an initial study there were significantly higher levels of baseline multifunctional $CD8^+$ cells in typhoid challenge participants who did not develop disease, in a subsequent study levels were higher in those who did develop disease (Fresnay et al. 2016, 2017). As well as producing IFN-γ, IL2, TNF-α and in some cases IL17A (McArthur and Sztein 2012), $CD8^+$ cells from participants vaccinated with Ty21a or CVD 908 kill *S.* Typhi infected cells (Salerno-Gonçalves, Rosângela Fernandez-Viña, Lewinsohn, and Sztein 2004; Salerno-Gonçalves et al. 2003; Sztein et al. 1995). Granzyme A and B, the serine protease effectors of cytotoxic T cell granules, are raised in the blood of typhoid fever patients compared with healthy controls, as

is the percentage of granzyme A-positive T cells and NK cells (de Jong et al. 2017). Inhibition of perforin by concanamycin A inhibits killing of *S.* Typhi infected cells, while anti-FasR antibody does not, suggesting that killing is granule-dependent (Salerno-Gonçalves, Rosângela Fernandez-Viña et al. 2004).

1.6.2 Non-classical T cells

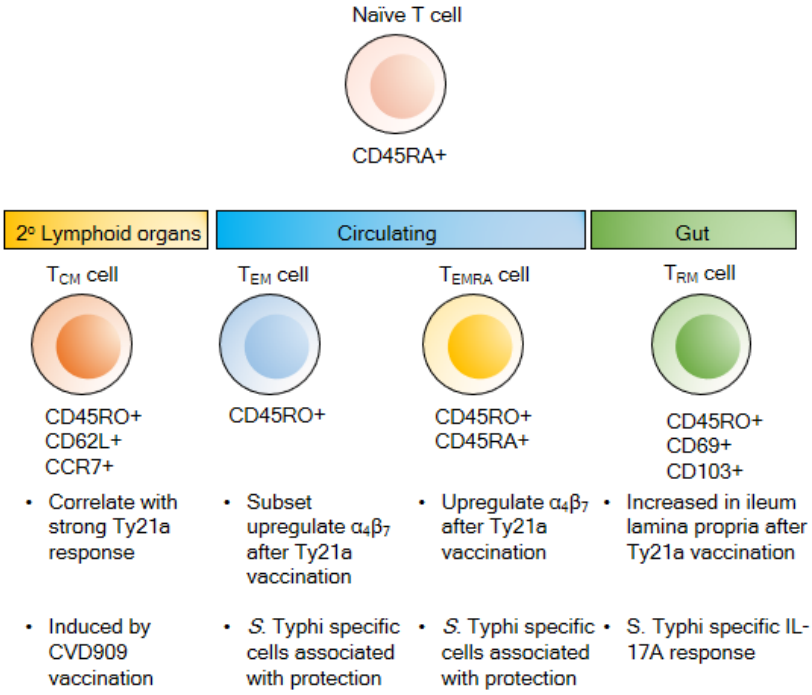
In addition to the recognition of classical MHC molecules, non-classical T cells which bind non-polymorphic antigen presenting molecules have also been identified as responsive to *S.* Typhi. Mucosal associated invariant T (MAIT) cells for example express an invariant TCR-α chain, through which they can recognise bacterial antigens presented on the conserved MR1 molecule (Gao and Williams 2015). Multiple TCR-β chains can be used, introducing some level of variability. Although *S.* Typhi induces little upregulation of MR1 in target cells upon infection, exposure to *S.* Typhi-infected cells induces MAIT cells to release IFN-γ, IL17a, and TNF-α (Salerno-Goncalves, Rezwan, and Sztein 2014). More recently, it has been found that while the number of MAIT cells significantly decreases in the peripheral blood during both acute typhoid and paratyphoid fever, the percentage of activated and proliferating $CD38^+$ MAIT cells increases (Fresnay et al. 2017; Howson et al. 2018). Furthermore, the composition of different TCR-β clonotypes changes during disease, suggesting that clonal expansion and contraction may take place. HLA-E restricted $CD8^+$ T cells may also play a role in enteric fever, having been found to specifically kill cells presenting *S.* Typhi antigens on this non-classical MHC Class I molecule (Salerno-Gonçalves, Rosângela Fernandez-Viña et al. 2004).

1.6.3 Memory T cells

Memory T cells, long-lived $CD45RO^+$ T cells which have encountered their cognate antigen and are able to respond more quickly upon re-exposure, are divided into several phenotypically distinct subsets: central memory T cells (T_{CM}) , which home to secondary lymphoid tissues; effector memory T cells (T_{EM}), which circulate in the periphery; and tissue-resident memory T

cells (T_{RM}), which remain at the site of infection (Willinger et al. 2005) (Figure 1.7). While most memory T cells downregulate naïve T cell marker CD45RA and upregulate CD45RO, a subset of T_{EM} cells, termed T_{EMRA}, are $CD45RA^+$.

Figure 1.7: Summary of T cell memory responses to *S.* Typhi infection or vaccination with live attenuated strains. T central memory (T_{CM}) cells reside in secondary lymphoid organs while T effector memory (T_{EM}) and CD45RA+ T effector memory (T_{EMRA}) cells are present in the circulation, and tissue-resident memory T cells (T_{RM}) in the gut. Surface markers which characterise these cell types are shown, as well as major findings relating to these cell types in typhoid infection or vaccination. Figure by A. Barton.



Both Ty21a vaccination and typhoid challenge result in multiple waves of increased $CD8^+$ memory cells in the blood (Fresnay et al. 2016; Salerno-Goncalves, Wahid, and Sztein 2010). This is attributed to expansion followed by migration to the gut; most T_{EMRA} cells express gut-homing integrin $\alpha_4\beta_7$ after vaccination, as do a subset of T_{EM} (Wahid et al. 2008). *S.* Typhi-specific $CD8^+$ memory cells are predominantly multifunctional, with greater multi-functionality amongst cells with gut-homing capacity (Fresnay et al. 2016). While there was a correlation between a strong, early T_{CM} response and a long-lived immune response in Ty21a vaccinees, in typhoid challenge participants high *S.* Typhi-specific $CD8^+$ T_{EM} and T_{EMRA} responses at baseline

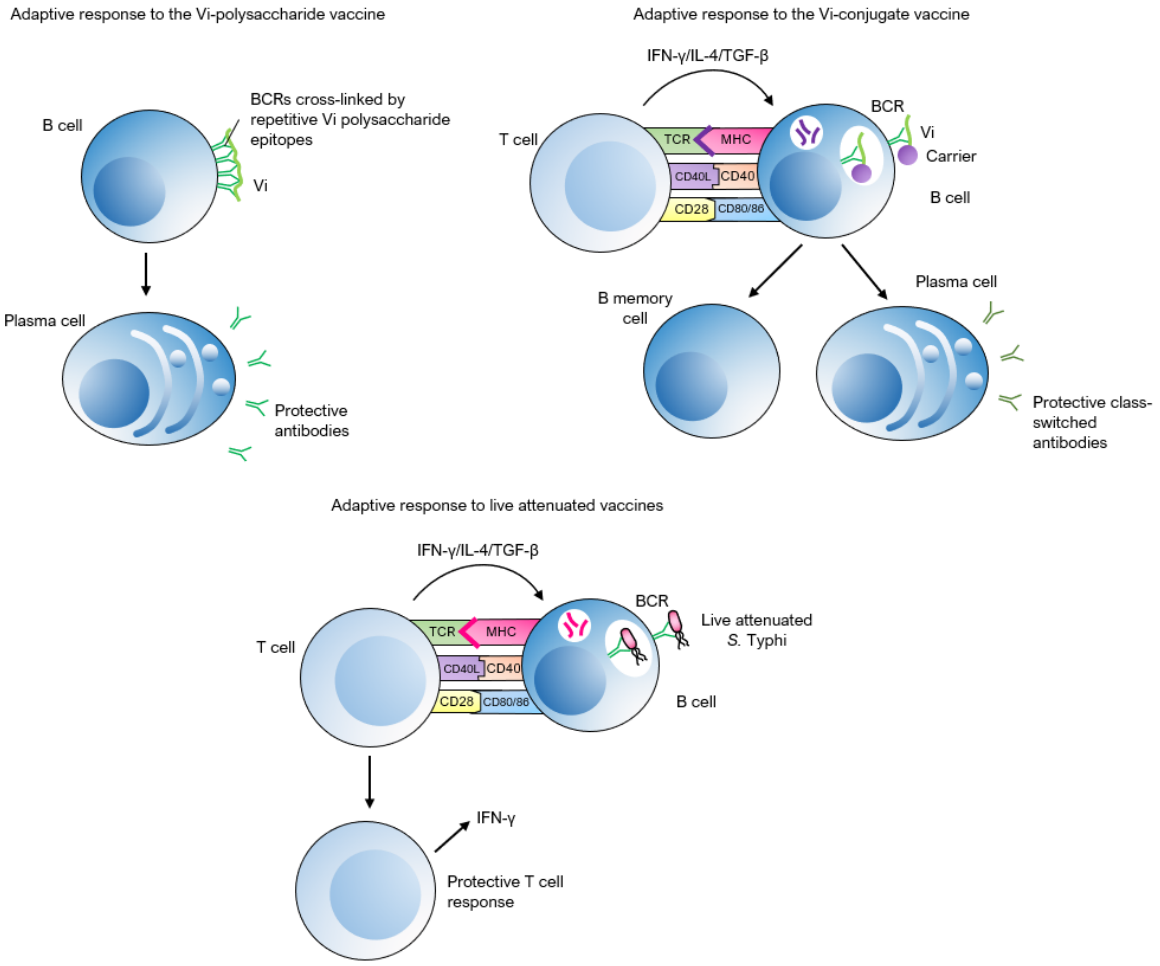
correlate with protection (Fresnay et al. 2016; Salerno-Goncalves et al. 2010). Whereas CVD 909 vaccination induces $CD4^+$ T_{EM} and T_{CM} which both secrete IFN-γ, after Ty21a vaccination few $CD4^+$ T_{CM} cells release cytokines (Wahid et al. 2008, 2016). Ty21a vaccination does however induce both multifunctional $CD4^+$ T_{EM} cells and a smaller population of multifunctional T_{EMRA} cells, of which one third express $\alpha_4\beta_7$ (Wahid et al. 2016). Recently, the proportion of T_{RM} cells positive for IFN-γ , IL17A or TNF-alpha has been found to be increased in the terminal ileum lamina propria following Ty21a vaccination, of which the IL17A response was identified as *S.* Typhi specific (Booth et al. 2019). Furthermore, Ty21a also induced higher IFN-γ, IL17A, IL2, and TNF-α expression in intra-epithelial T_{RM} cells, although this was not antigen-specific.

1.6.4 B cells

B cell responses to different *S.* Typhi vaccine antigens are summarised in Figure 1.8. As a polysaccharide antigen, the Vi capsule cannot be presented by the MHC complex, and likely bypasses T cell help by crosslinking B cell receptors (BCRs) on Vi-specific B cells, resulting in activation (Mak and Saunders 2005; Pandya et al. 2018). Despite being T cell independent, vaccination with the Vi antigen is strongly protective, with efficacy estimates from field studies of approximately 87% (Yang et al. 2001). Thus, the B cell response likely plays an important role in adaptive immunity to enteric fever. In the case of Vi antigen, Vi antibodies themselves appear to mediate protection: opsonisation of Vi-expressing *S.* Typhimurium with Vi antiserum reduces invasion when used to challenge naïve mice (Marshall et al. 2012). In both naïve and Vi-vaccinated human challenge participants, higher Vi titres were associated with resistance to infection, although this was not the case within the group which received the Vi-tetanus toxoid conjugate vaccine (Jin et al. 2017; Waddington, Darton, Jones, et al. 2014). Despite the protective capacity of Vi antibodies, acute typhoid infection does not increase Vi antibody titres in the human challenge model (Waddington, Darton, Jones, et al. 2014). However in Fiji, Vi antibody seropositivity prevalence increases with age, which would suggest that frequent exposure does build up an anti-Vi response (Watson et al. 2017). Although the Vi-polysaccharide vaccine

induces reasonable albeit short-lived anti-Vi responses in adults, young children, the main target of *S.* Typhi infection, are unable to mount a robust B cell response to the Vi capsule polysaccharide (Dickinson et al. 2018). This may be due to the lack of IL7-dependent B cell lymphopoiesis compared with adults, or the lack of a mature splenic marginal zone (Clutterbuck et al. 2008). Thus it is thought that by inducing T cell help, the Vi-tetanus toxoid conjugate vaccine could be more efficacious in children.

Figure 1.8: Role of B cells in vaccine-mediated protection. The Vi-polysaccharide vaccine stimulates secretion of protective Vi antibodies by plasma cells in a T cell-independent manner. In the case of Vi-conjugate vaccines, presentation of peptides from the carrier protein to T cells induces T cell help, resulting in production of memory B cells and class-switching. Following a live attenuated typhoid vaccine, presentation of bacterial peptides by B cells activates a protective T_H1 response. Figure by A. Barton.



In human challenge participants there is a rise in activated plasmablasts during acute disease (Toapanta et al. 2016). Although acute infection does not increase Vi antibody titres, both oral vaccination and human challenge with *S.* Typhi or *S.* Paratyphi A result in a rise in LPS antibody (Darton et al. 2016; Dobinson et al. 2017; Waddington, Darton, Jones, et al. 2014). Participants with greater LPS antibody at baseline are more resistant to *S.* Paratyphi A infection, while no such pattern was seen for *S.* Typhi challenge, possibly as *S.* Paratyphi A LPS is more exposed. LPS antibodies are capable of mediating complement-dependent *S.* Typhi killing *in vitro*, but binding is reduced in the presence of the Vi capsule (Hart et al. 2016). While LPS antibody titre in humans vaccinated with Ty21a or CVD 909 correlates with antibody dependent phagocytosis, no such relationship has been identified between LPS antibody and phagocytic killing (Wahid et al. 2014).

In addition to producing antibodies, B cells can help drive T cell responses. This interaction appears to play a key role in oral vaccine mediated immunity. Although not professional phagocytes, human primary B cells are able to take up *S.* Typhimurium bound to their BCR (Souwer et al. 2009). *S.* Typhimurium uptake induces B cell differentiation and secretion of *Salmonella* specific antibodies, but also results in antigen presentation by MHC Class I and II molecules, activating CD4+ and CD8+ T cells (Souwer et al. 2009; de Wit et al. 2010). When vaccinated with an attenuated strain of *S.* Typhimurium, wild-type mice were protected against subsequent *S.* Typhimurium challenge, while B cell-deficient mice were unable to survive (Mastroeni et al. 2000). However, injection of B cell-deficient mice with serum from immune mice did not improve their outcome, suggesting it was the impaired T_H1 response observed in the B cell-deficient mice rather than lack of antibody which prevented a protective secondary response. Furthermore, transgenic mice that possess B cells which cannot secrete antibody are only slightly more susceptible to *S.* Typhimurium challenge than wild-type mice (25% mortality, compared with 100% in the B cell deficient group, and none in the wild-type group) (Nanton et al. 2012).

The contribution of mucosal humoral responses to enteric fever protection has yet to be elucidated. However, in human challenge participants a drop in peripheral B cells and a rise in B cell α4β7 expression is observed during acute disease, suggesting they may be homing to the gut (Toapanta et al. 2016). Furthermore, high Vi antibody titres are associated with a lower likelihood of shedding live bacteria in the stool after challenge (Gibani, Voysey, et al. 2019).

1.7 The carrier state

During the bacteraemia associated with acute disease, the gallbladder is a major site of secondary infection (Gonzalez-Escobedo, Marshall, and Gunn 2011). The presence of bile induces transcriptional changes in *S.* Typhi, resulting in upregulation of the anti-oxidative enzymes superoxide dismutase and catalase by a mechanism dependent on quorum sensing (Walawalkar, Vaidya, and Nayak 2016). Bile also induces *S.* Typhi but not *S.* Typhimurium to upregulate SPI-1 genes, increasing invasion of the gallbladder epithelium (Byrne et al. 2017).

An estimated 2-4% of those with acute enteric fever go on to become chronic carriers, shedding bacteria from the gallbladder into their faeces and acting as a human reservoir of infection (Dougan and Baker 2014). Gallstones are a major risk factor for chronic carriage, affecting an estimated 90% of carriers (Lovane et al. 2016). Mice fed a high-cholesterol diet to induce gallstone formation had greater bacterial loads in the gallbladder and faeces when challenged with *S.* Typhimurium, despite equal colonisation of the spleen (R. W. Crawford et al. 2010). Both *S.* Typhi and *S.* Typhimurium form biofilms on the surface of cholesterol gallstones in the presence of bile, giving rise to a thick, loosely packed cell matrix connected by a web of proteins, polysaccharides and extracellular DNA (Adcox et al. 2016; Prouty, Schwesinger, and Gunn 2002). This process is dependent on both quorum sensing, allowing the bacteria to sense their population density, and flagella, which provide a scaffold to which other bacteria can bind (Robert W. Crawford, Reeve, and Gunn 2010; Walawalkar et al. 2016). Biofilms can be directly visualised by electron microscopy on the surface of gallstones from human *S.* Typhi carriers,

and are thought to render the bacteria resistant to antibiotic treatment (R. W. Crawford et al. 2010).

Although the Vi antigen is not necessary for biofilm formation, it does constitute part of the extracellular biofilm matrix (Adcox et al. 2016; Prouty et al. 2002). Among those infected with *S.* Typhi, carriers constitute the few who raise substantial Vi antibody responses (Dougan and Baker 2014). The typhoid toxin has emerged as a potentially more important virulence factor affecting chronic carriage, its expression in transgenic *S.* Typhimurium increasing the proportion of asymptomatic carriers following murine challenge (Del Bel Belluz et al. 2016).

1.8 Aims and Objectives

There is a considerable amount of literature investigating *in vitro* interactions between *S.* Typhi and the "first responders" of enteric immunity: the monocytes and the intestinal epithelium. Likewise, much work has been carried out to investigate *in vivo* interactions between mice and *S.* Typhimurium. However, early responses to *S.* Typhi and *S.* Paratyphi A infection *in vivo* in humans are poorly characterised, as enteric fever sufferers do not clinically present until secondary bacteraemia. It is also unknown why in the absence of vaccination, following the same exposure, one person exposed to *S.* Typhi might develop acute disease, while another remains healthy. This thesis has three major aims:

1.8.1 Characterising early host responses to infection

Human host responses to infection with *S.* Typhi and *S.* Paratyphi A during the period associated with primary bacteraemia will be characterised by:

- Detailed profiling of early plasma cytokine responses to human challenge with *S.* Typhi using multiplex cytokine assays

- Identification of the leukocyte subsets responsible for secreting “early response” cytokines and bacterial uptake by *in vitro* infection with typhoidal *Salmonella* strains
- Whole blood transcriptional profiling after *S.* Typhi and *S.* Paratyphi A challenge using microarray and RNA-sequencing, in the presence and absence of vaccination

1.8.2 Genetic risk factors for enteric fever

Risk factors for the development of acute disease following exposure to *S.* Typhi and *S.* Paratyphi A will be investigated by relating outcome of infection with:

- The genotype of participants undergoing *S.* Typhi challenge
- HLA types of participants undergoing both *S.* Typhi and *S.* Paratyphi A challenge
- Gene expression at baseline and early responses to infection

1.8.3 Correlates of fever and symptom score

Human challenge models present a unique opportunity to investigate host factors relating to severity without confounding variation in pathogen dose or strain. Correlates of symptomology and inflammation will be identified by:

- Correlation of plasma cytokine profiles in acute disease with body temperature, C-reactive protein and symptom score
- Association of HLA types with maximum temperature during acute disease

Chapter 2 | Human challenge cohorts

The following chapter will give an outline of both historical typhoid human challenge studies, which provided early insights into the pathogenesis of enteric fever, and contemporary human challenge studies, from which the samples analysed for the purpose of this thesis were derived.

2.1 The history of typhoid human challenge models

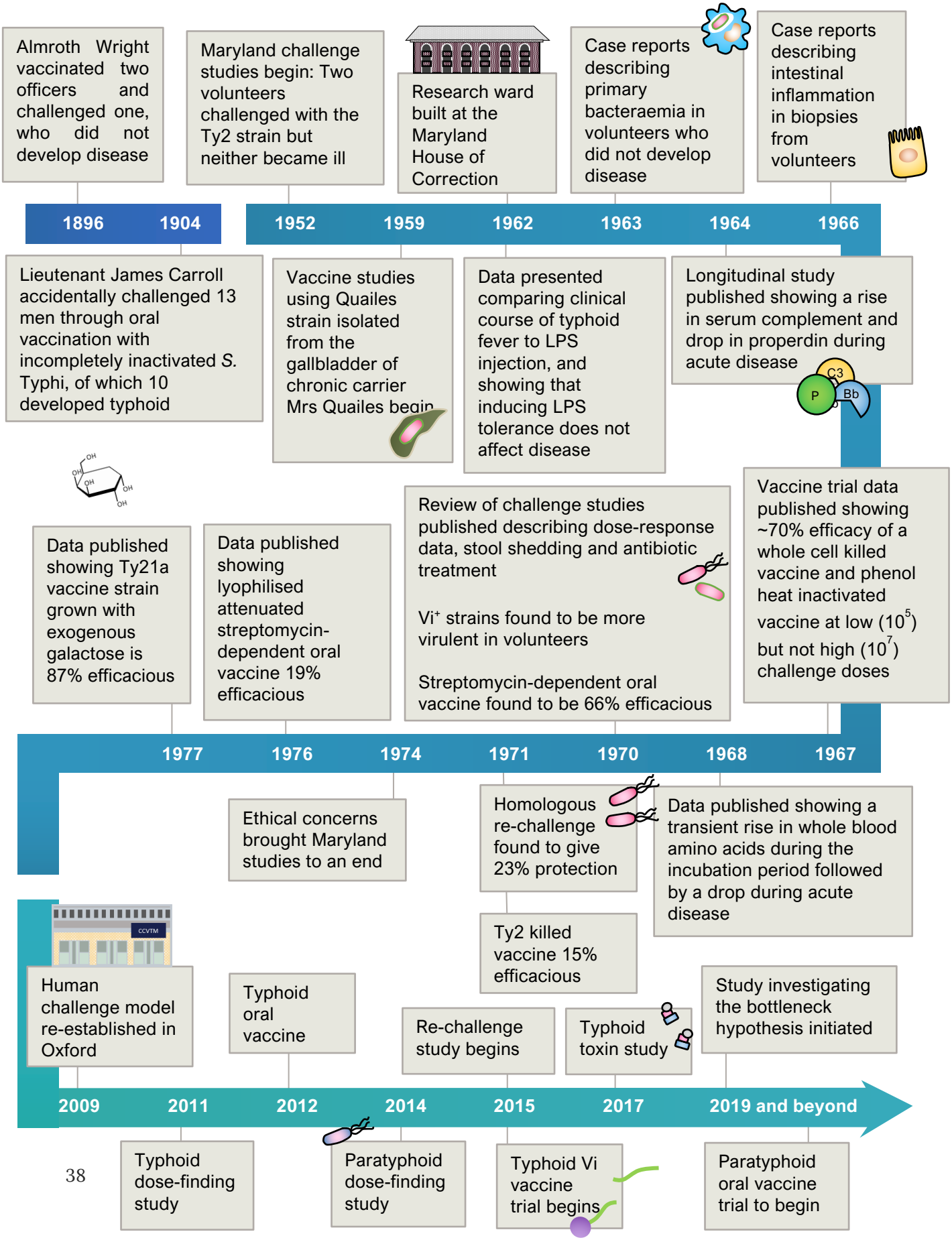
The first recorded typhoid human challenge took place over 100 years ago in 1897, when British microbiologist Almroth Wright vaccinated two officers from the Indian Medical Service with killed *S.* Typhi, then injected one with "more virulent" *S.* Typhi two weeks later, resulting in no disease (Wright 1896). The second reported human challenge took place unintentionally, as James Carroll from the US Army Medical school vaccinated volunteers with incompletely heat-killed bacteria, accidentally giving ten men (including himself) typhoid fever (Woodward 1980).

In the 1950s a human challenge model was established by Theodore Woodward at the University of Maryland (results summarised in Figure 2.1). This involved suspending *S.* Typhi Quailes strain, isolated from the gallbladder of chronic carrier Mrs Quailes, in milk, and orally challenging prisoners from the Maryland House of Correction. As well as providing a method of assessing vaccines, including the first licensed typhoid vaccine Ty21a, the model gave novel insights into the pathogenesis of typhoid fever. This included the finding that higher doses resulted in higher attack rates and shorter incubation periods; the presence of a primary bacteraemia within the first 24 hours after infection; a relationship between enteritis and disease severity but no association between enteritis and gastrointestinal symptoms; and the presence of a transient rise in properdin and amino acids in the blood during the incubation period (Waddington, Darton, Woodward, et al. 2014).

Typhoid challenge studies in Maryland came to an end in 1974 following a lawsuit from the American Civil Liberties Union. However in 2011 a human typhoid human model was established

in the Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford. Although using the same Quailes strain, bacteria are suspended in sodium bicarbonate to neutralise gastric acid, and challenge is carried out on adult volunteers in an outpatient setting. The primary outcomes of the studies performed in Oxford to date are shown in Figure 2a-f.

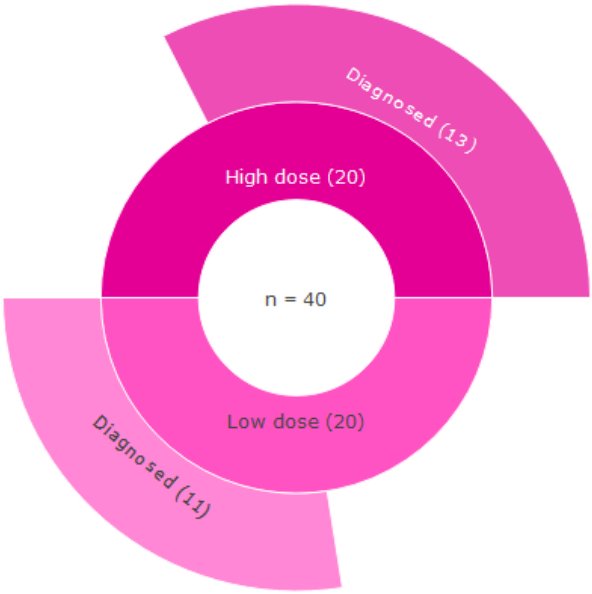
Figure 2.1: Summary of the history of typhoid human challenge models and major findings from the studies which took place in Maryland (Dupont et al. 1971; Feigin et al. 1968; Hornick et al. 1970; Hornick and Woodward 1967; Schubart et al. 1964; Snyder et al. 1963; Sprinz et al. 1966; Waddington, Darton, Woodward, et al. 2014; Woodward 1980). Figure by A. Barton.



2.2 Oxford human challenge cohorts

Figure 2a.

2.2.1 Typhoid dose-finding study (Waddington, Darton, Jones, et al. 2014)

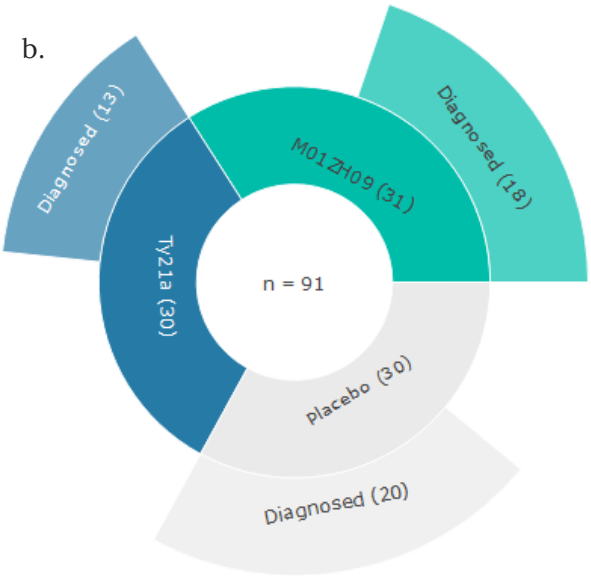


As the first human challenge model to be carried out since 1974, the aim of this study was to find the inoculum dose which gave an attack rate of 60-75% with *S.* Typhi Quailes strain suspended in sodium bicarbonate. A diagnosis was made where participants recorded an oral temperature ≥38°C sustained ≥12 hours or a positive blood culture. After either being diagnosed with typhoid or remaining healthy for 14 days, participants were administered 500mg ciprofloxacin twice daily for 14 days. An extension study took place in which control participants drank bicarbonate only, and plasma samples were taken at baseline, 12 hours post-challenge and 24 hours post-challenge.

At an initial dose of 1-5 x 10^3 CFU 55% (11/20) of participants were diagnosed. Escalating the dose to 1-5 x 10^4 CFU gave an attack rate of 65% (13/20), satisfying the primary endpoint.

2.2.2 Typhoid oral vaccine trial (Darton et al. 2016)

b.



The aim of this trial was to compare the protective efficacy of one dose of the attenuated oral vaccine candidate M01ZH09 with three doses of Ty21a or a placebo suspension. 28 days after vaccination participants were challenged with 1 – 5 x 10^4 CFU *S.* Typhi Quailes strain. Diagnostic criteria were adjusted to an oral temperature ≥38°C sustained ≥12 hours after day 5 post-challenge, positive blood culture after day 7 post-challenge or positive blood culture after day 5 post-challenge plus symptoms of typhoid. After diagnosis or 14 days participants received either azithromycin or ciprofloxacin.

Compared with placebo, the vaccine efficacy of one dose of M01ZH09 was 13%, and the efficacy of three doses of Ty21a was 35%. At a confidence interval of 95% neither vaccine was significantly protective against disease.

2.2.3 Paratyphoid dose-finding study (Dobinson et al. 2017)

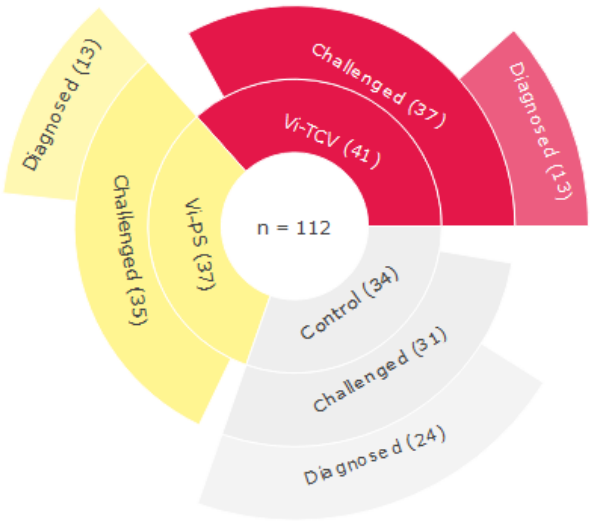
c.



The aim of this study was to find the inoculum dose of *S.* Paratyphi A NVGH308 strain giving an attack rate of 60-75%. Participants were diagnosed based on an oral temperature ≥38°C sustained ≥12 hours or a positive blood culture after 72 hours post-challenge. After diagnosis or 14 days participants were administered ciprofloxacin.

The primary objective was met at an initial dose of 1–5 × 10^3 CFU, giving an attack rate of 60%. At a slightly lower dose of 0.5–1.0 × 10^3 CFU the attack rate was 40%.

d.

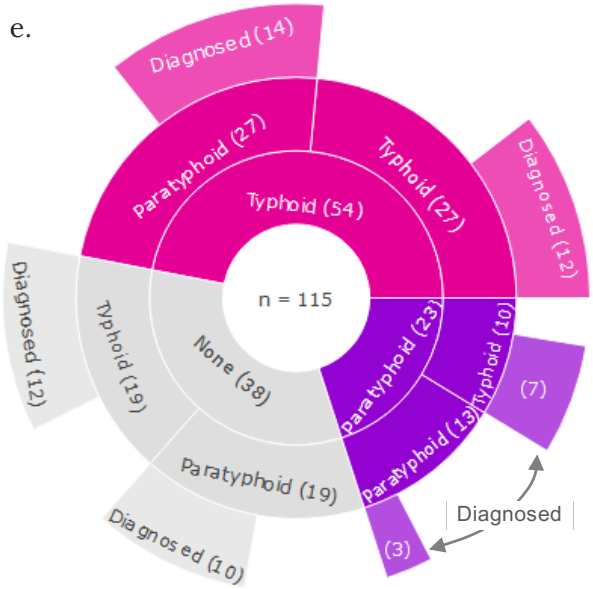


2.2.4 Typhoid Vi-vaccine trial (Jin et al. 2017)

The aim of this study was to compare the efficacy of the Typbar TCV conjugate vaccine (Vi-TCV, consisting of the Vi polysaccharide conjugated to tetanus toxoid) to that of the licensed Vi-polysaccharide vaccine (Vi-PS). Control participants received a meningococcal ACWY-CRM conjugate vaccine. One month after vaccination participants were challenged with 1–5 × 10^4 CFU *S.* Typhi Quailes strain. Participants were diagnosed based on an oral temperature ≥38°C sustained ≥12 hours or positive blood culture after 72 hours post-challenge. After diagnosis or 14 days participants were treated with either ciprofloxacin or azithromycin.

Comparison with the control group indicated an efficacy of 54.6% for the Vi-PS vaccine and 52% for the Vi-TCV vaccine. Both vaccines were significantly protective against disease. Following the initial study a subset of participants who received the Vi-TCV vaccine were vaccinated with Vi-PS to assess booster responses.

e.



2.2.5 Re-challenge study (Gibani et al. 2017)

The aim of this study was to compare the attack rate in naïve participants with those who had been challenged previously with either *S.* Typhi or *S.* Paratyphi A. Participants in each of these three groups were randomised to undergo either *S.* Typhi or *S.* Paratyphi A challenge. A further group of 10 participants received sodium bicarbonate only. Participants were diagnosed based on an oral temperature ≥38°C sustained ≥12 hours, positive blood culture after 72 hours post-challenge, positive blood culture within 72 hours post-challenge plus symptoms of enteric fever or persistent positive blood cultures within 72 hours post-challenge. After diagnosis or 14 days participants received ciprofloxacin.

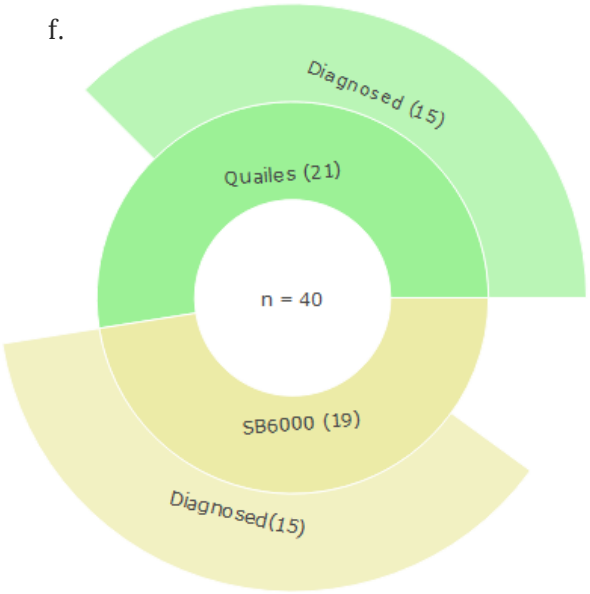
Overall a reduction in attack rate was seen following re-challenge, the largest reduction being in those undergoing homologous *S.* Paratyphi A challenge (23% diagnosed following re-challenge, as opposed to 67% on the first exposure). There was no trend towards protection in the *S.* Paratyphi A-*S.* Typhi group (70% attack rate). In those who had previously undergone challenge with *S.* Typhi, the attack rate was marginally lower compared with naïve individuals (50% attack rate in the *S.* Typhi-*S.* Typhi group, 53% attack rate in the *S.* Typhi-*S.* Paratyphi A group, and 65% attack rate in the naïve group). However no differences between groups were statistically significant, likely due to the small sample size in each group.

2.2.6 Typhoid toxin study (Gibani, Jones, et al. 2019)

The aim of this study was to compare the attack rate between *S.* Typhi Quailes strain and an isogenic typhoid toxin knockout strain SB6000, in order to assess the role of the typhoid toxin

in establishing acute disease. Participants were diagnosed based on an oral temperature ≥38°C sustained ≥12 hours or positive blood culture. After diagnosis or 14 days participants received ciprofloxacin.

f.



Absence of the typhoid toxin did not significantly alter the attack rate, with 71% diagnosed in the Quailes strain group and 79% in the SB6000 knock-out group. However the average duration of bacteraemia was significantly higher in the group challenged with the knockout strain.

2.2.7 Upcoming studies

The Bottlenecks study, initiated in June 2019, aims to explore whether bacteraemia arises from one founder organism or multiple bacteria overcoming the barrier to infection. Participants will be challenged with a mixture of Quailes and SB6000 *S.* Typhi, and PCR carried out on colonies from blood CFU counts to identify whether bacteraemia is mixed or single strain. An *S.* Paratyphi A attenuated oral vaccine trial is expected to be underway in 2020.

2.3 The advantages of human challenge studies

Typhoid human challenge models have many unique advantages over field studies (see Table 2.1): factors such as dose, strain and timing can be controlled; host-pathogen interactions during the incubation period can be investigated; presence of a paired baseline sample increases statistical power, and vaccine efficacy can be assessed quickly. As demonstrated by the Typhoid toxin study, the challenge agent can even be genetically modified to investigate the function of virulence factors (Gibani, Jones, et al. 2019).

Mouse models of infection involve either oral challenge with *S.* Typhimurium (Dougan and Baker 2014), peritoneal injection of *S.* Typhi (Gerichter 1960), or rendering the host susceptible through iron overload (Ghosh et al. 2011), genetic manipulation or humanisation (Mathur et al. 2012). In human challenge studies however, immunocompetent humans are challenged with the serovar of interest, and the contribution of natural genetic variation to disease susceptibility can be determined.

Nonetheless, human challenge models do have a number of limitations. Firstly, they take place in a naïve and predominantly Caucasian population, rather than in the populations naturally exposed to disease. In future, typhoid human challenge may take place in endemic settings such as Malawi (Gordon et al. 2017; Raymond et al. 2019). Secondly, human challenge studies are limited in size compared with field studies. As an antibiotic-susceptible strain isolated in the 1960s, challenge with Quailes strain does not capture the breadth and virulence of contemporary *S.* Typhi, while the short incubation period observed in the human challenge model suggests that volunteers are likely exposed to a higher dose than in a natural setting.

Table 2.1: Comparison of different approaches to enteric fever research

Ability to:	Mouse challenge model	Human challenge model	Human field study
Recapitulate human disease e.g. serovar, route of infection			
Control for pathogen dose and strain			
Control other environmental factors			
Research large samples			
Study disease in an endemic setting with non-naïve participants			
Genetically manipulate subjects			
Genetically manipulate the pathogen			
Associate natural genetic variants with susceptibility to infection			
Study early and asymptomatic infection			
Study severe disease e.g. intestinal perforation, death			
		Difficult/Impossible  Easy/Common	

2.4 Samples analysed in this thesis

The work presented in this thesis explores changes in plasma cytokine profile post-challenge, changes in blood transcriptome early after challenge, and genetic susceptibility to infection. Table 2.2 outlines the main cohorts used for each analysis, and Figure 2.3 the samples from each participant for the main datasets used.

Table 2.2: Cohorts used for each analysis/experiment		
Analysis	Discovery cohorts	Validation cohorts
Changes in plasma cytokine profile	Typhoid toxin study	Typhoid dose escalation Typhoid Vi vaccine trial Typhoid toxin study (different platform) Typhoid dose escalation bicarbonate sub-study
Changes in blood transcriptome	Typhoid dose escalation Typhoid oral vaccine trial	Paratyphoid dose escalation Typhoid Vi vaccine trial
Ex vivo changes in neutrophil activation	Typhoid toxin study	
SNP association	Typhoid dose escalation Typhoid oral vaccine trial	Vietnam case-control study
HLA type association	Typhoid dose escalation Typhoid oral vaccine trial Paratyphoid dose escalation Typhoid Vi vaccine trial	
Expression quantitative trait loci	Typhoid toxin study Typhoid dose escalation	

Figure 2.3: Samples processed and analysed from each cohort for the purpose of this thesis. Sample types and time points are shown in the y axis and participants on the x axis. Samples are coloured green if included in the analysis, pink if processed but excluded during to quality control, and grey if either unavailable or not processed for the purpose of this work. Participants are annotated by challenge strain, typhoid vaccine received, outcome of challenge and dose.



2.4 Conclusion

In the past, typhoid human challenge models have made a major contribution to the understanding of enteric fever, and provided evidence of efficacy for both the Ty21a and Vi-TCV vaccines. In the following chapters, we make use of these major advantages to investigate early host responses in detail, and identify potential genetic risk factors for the development of acute disease.

Chapter 3 | Early and Acute Plasma Cytokine Responses to Wild-type and Toxin-Negative *Salmonella* Typhi

3.1 Introduction

As the mechanism by which immune cells communicate with one another, cytokines play a major role in the host response to infection, affecting immune cell differentiation, activation and chemotaxis (see Table S3.1). In *S.* Typhi this modulation is often ascribed to the SPI-7 locus, expression of which downregulates flagellin and upregulates synthesis of the polysaccharide Vi capsule following intestinal invasion (Raffatellu et al. 2005). In doing so, *S.* Typhi is able to avoid activation of TLRs and therefore reduce production of neutrophil chemoattractant IL8, preventing inflammatory diarrhoea and allowing systemic dissemination (Wilson et al. 2011). In human challenge participants, sCD40L, fractalkine, GROα, IL1RA, EGF and VEGF are raised 12 hours post-challenge, the time point around which *S.* Typhi is thought to invade the intestinal epithelium (Blohmke et al. 2016). The origin of this signal is unknown: although *ex vivo* stimulation of intestinal biopsies with *S.* Typhi induces production of IFN-γ, IL10, IL1β, IL2 and IL4, these cytokines are only significantly released at the apical surface (Nickerson et al. 2018).

During acute typhoid disease, plasma levels of IFN-γ, TNF-α, IP-10, TNF-R and IL6 are raised relative to healthy controls (Blohmke et al. 2016; Butler et al. 1993). However, compared with invasive non-typhoidal *Salmonella* disease the levels of inflammatory cytokines in the plasma are much lower, despite a similar bacterial load of 1 CFU/ml (Gilchrist et al. 2016; Gordon et al. 2010). Although the immuno-suppressive Vi capsule is thought to play a role (R. P. Wilson et al. 2008), given that *S.* Paratyphi A lacks the Vi capsule but cause a similar clinical syndrome to *S.* Typhi, non-Vi factors must also differentiate typhoidal and non-typhoidal serovars. One such candidate is the typhoid toxin, which induces cell cycle arrest through DNA damage and is expressed by both *S.* Typhi and *S.* Paratyphi A (Galán 2016).

In addition to field and human challenge samples, several *in vitro* models have previously been used to characterise cytokine responses to *S.* Typhi and *S.* Typhimurium infection. For interactions with white blood cells, models have generally been limited to stimulation of primary PBMCs (Wyant et al. 1999) or immortalised monocyte-like cell line THP-1 (Raffatellu et al. 2005). As primary cells from the human intestinal epithelium are less accessible, and the intestinal mucosa has a complex three-dimensional structure consisting of many cell types, a variety of models have emerged. These have included epithelial cell lines (Kuzminska-Bajor, Grzymajlo, and Ugorski 2015), intestinal biopsies (Nickerson et al. 2018), primary organoids (Zhang et al. 2014) and three dimensional organotypic models (Salerno-goncalves et al. 2019). Induced intestinal organoids, an emerging model in which induced pluripotent stem cells are differentiated into a spherical epithelium, have the advantage of containing a variety of cell types (enterocytes, goblet cells, paneth cells), being three dimensional and being genetically tractable (Lees et al. 2019).

Previous literature investigating cytokines in typhoidal *Salmonella* infection is outlined in Table 3.1. While human cytokine responses in acute typhoid fever have been previously studied, it is not currently known how cytokine responses relate to symptomology, and only a limited number of cytokines have been measured early after challenge. Furthermore, as mouse and *in vitro* models have focused on production of IL8 and inflammatory cytokines, it is not known which cells might produce “early response” cytokines.

Table 3.1: Published literature assessing changes in cytokines in the plasma in enteric fever, murine *S.* Typhimurium infection, and *in vitro* typhoidal *Salmonella* stimulation				
Human studies				
Cohort	Cytokines	Timepoints	Finding	Reference
Adult Nepalese patients with culture-positive typhoid fever (n = 29)	IL6, IFN-γ, TNF-R	Acute disease, before treatment, and on days 4 and 15 after start of treatment	IL6, IFN-γ and TNF-R were raised relative to healthy controls and fell over the course of treatment. Mean levels of IL6 and TNF-R p55 before treatment were higher in those who suffered from prolonged fever or relapse.	(Butler et al. 1993)
Adult Indonesian hospital patients with culture-positive typhoid fever (n = 44)	TNFα, IL1β, IL6, IL8, sTNF-R, TNFβ, IL1RA	On admission (acute disease) and convalescence	IL1RA and sTNF-R (p55 and p75) were higher in acute disease than convalescence. No differences were found between those with complicated and uncomplicated typhoid fever.	(Keuter et al. 1994)
Typhoid dose finding human challenge study (n = 41)	sCD40L, CX_3CL1, VEGF, EGF, IL1RA, GROα, IL8, MIP-1β, TNFα, IFN-γ, IP10, IL6, IL12, IL1α, IL2, IL10, IL17, Flt-3L	Baseline, 12, 24 and 48 hours post-challenge; 96, 46 and 24 hours before diagnosis with acute disease; 48 and 96 hours following diagnosis; Day 14	TNFα, IFN-γ and IP10 were raised in acute disease. sCD40L, CX_3CL1, VEGF, EGF, IL1RA and GROα were raised 12 hours post-challenge, returning to baseline by 24 hours post-challenge.	(Blohmke et al. 2016)
Typhoid toxin human challenge study (n = 40)	62-plex: See table S3.1	Baseline, 12, 24 and 48 and 96 hours post-challenge, 24-48 hours before typhoid diagnosis, time of typhoid, 12-24 hours after diagnosis; Day 14	The following thesis chapter	
Mouse models of invasive *Salmonella* infection				
Male C57BL/6J mice infected intraperitoneally with *S.* Typhimurium (n = 63)	TNFα, IL6, IFN-γ and IL-lα	1, 3, 6, 9, 12 hours post-inoculation; 1, 2, 3 and 4 days post-inoculation	TNFα and IL6 increased from day 1 to day 4, while IFN-γ showed a transient peak at day 3.	(Jotwani et al. 1995)
Tlr11$^{-/-}$ mice orally administered *S.* Typhi (n = 5)	IL12p40, TNFα, IL6	Day 8-10 post-infection	IL-12p40 and TNFα were raised in the serum of *Tlr11*$^{-/-}$ mice relative to uninfected controls and wild-type mice.	(Mathur et al. 2012)
IL-10$^{-/-}$ and wild-type C57BL/6 mice infected orally with *S.* Typhimurium (n not specified)	IL-1β, IL6, IL10, IFN-γ, IL12p70, IL23p19, and TNFα	Day 5 post-infection	The only cytokine raised in the serum of infected wild-type mice was IL10. IL10$^{-/-}$ mice had higher IFN-γ, IL6, TNFα, IL12, and IL23.	(Salazar et al. 2017)
In vitro models of typhoidal *Salmonella* infection				

Cell types	Cytokines	Conditions	Findings	Reference
T84 (epithelial cell line) and THP-1 (macrophage-like cell line)	IL8	MOI 20-25 T84 infected 2.5 hours, THP-1 cells for 6 hours	Vi-encapsulated *S.* Typhi elicited lower IL8 secretion than non-encapsulated.	(Winter et al. 2008)
Intestinal biopsies	IFN-γ, IL10, IL12, IL13, IL1β, IL2, IL4, IL8, IL6, TNFα	10^8 CFU per biopsy Up to 2 hours	*S.* Typhi inducds production of IFN-γ, IL10, IL1β, IL2 and IL4 at the apical surface.	(Nickerson et al. 2018)
3-D intestinal organotypic model	IL1β, IL6, IL8, CCL3, and TNFα	MOI 100 Up to 6 hours	S. Paratyphi B induced greater secretion of IL6 and TNF-α than S. Paratyphi A or S. Typhi, while S. Paratyphi A induced greater secretion of CCL3.	(Salerno-goncalves, Kayastha, and Fasano 2019)
All leukocytes, monocytes, lymphocytes, granulocytes, intestinal organoids	CD40L, EGF, GROα, IL1RA, IL2, IL6, IL8, TGFα, TNFα	MOI 10 2,4 or 8 hours	The following thesis chapter	

This chapter presents the most detailed characterisation of the temporal cytokine response to typhoid infection to date, including the response to challenge with a toxin-negative *S.* Typhi strain. BDNF is identified as a novel 'early response' cytokine, and MIG, IP10 and IL1RA as potential biomarkers of severity in acute disease. Furthermore, we identify many of the early response cytokines as being secreted by primary white blood cells in response to *S.* Typhi stimulation, particularly by granulocytes and monocytes.

3.1.1 Research questions

In the following chapter we attempt to address the following research questions with regards to plasma cytokine responses in enteric fever:

1. Question: Given that sCD40L, fractalkine, GROα, IL1RA, EGF and VEGF have previously been identified as raised 12 hours post-challenge, are there any further early response cytokines which may give insight into host-pathogen interactions?

Approach: A 62-plex assay is used to simultaneously screen for changes in as many plasma cytokines as possible. Cytokines identified as raised 12 hours post-challenge are down-selected and measured using an independent assay for validation.

2. Question: Does this cytokine signal arise from the intestinal epithelium, or from a particular leukocyte subset?

   Approach: As intestinal biopsies and PBMCs from human challenge participants were not available at this early timepoint for intracellular cytokine staining, intestinal organoids, monocytes, lymphocytes and granulocytes were stimulated with *S.* Typhi *in vitro* to assess their capacity to produce these cytokines when infected.

3. Question: Given that *S.* Typhi has been previously identified as less inflammatory in acute disease and *in vitro* than *S.* Typhimurium, which is generally attributed to the Vi capsule, how does *Salmonella* serovar and presence of the Vi capsule affect the secretion of these early response cytokines?

   Approach: White blood cells were stimulated with a variety of *Salmonella* serovars and strains in order to dissect the role of serovar and the Vi capsule in inducing early response cytokines.

4. Question: Which cytokines are raised during acute disease, and do they correlate with fever or other symptoms?

   Approach: As above, a 62-plex assay is used to simultaneously screen for changes in as many plasma cytokines as possible. Linear modelling is used to identify the cytokines most raised in acute disease. These cytokines are then correlated with oral temperature, symptom score and CRP, a measure of inflammation. Finally, we retrospectively analyse data from previous studies to validate this association in an independent cohort.

5. Question: Can the (non-significantly, p = 0.3) higher incidence of “severe” typhoid (oral temperature ≥40°C or grade 4 laboratory abnormality) in the toxin-negative group be attributed to a difference in cytokine profiles?

   Approach: We screen for differences between groups in as many plasma cytokines as possible over the entire course of disease and at individual timepoints.

3.2 Materials and methods

3.2.1 Study design

Samples were collected from the typhoid toxin study (see Chapter 2.2.6). 40 participants were randomised to receive oral challenge with 1-5x10^4 CFU wild type *S.* Typhi (Quailes strain) or a typhoid toxin-deficient isogenic mutant (strain SB6000). Participants were diagnosed with typhoid if they presented with an oral temperature ≥38.0°C persisting for 12 hours, a positive blood culture from 72 hours after challenge, persistent positive blood cultures for *S.* Typhi within 72 hours of challenge, or a positive blood culture for *S.* Typhi within 72 hours of challenge combined with symptoms of infection. 30/40 participants met the diagnostic criteria, with an attack rate of 71% (15/21) in the wild-type group and 79% (15/19) in the toxin-negative group. Those with an oral temperature ≥40°C or grade 4 laboratory abnormality were considered to have severe disease (1/21 in the wild-type group and 4/19 in the toxin-negative group). At certain time points participants scored each of their symptoms, such as headache, abdominal pain or diarrhoea, from 0-4 (0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = life-threatening or requiring hospitalisation), and individual symptom scores were summed to give a cumulative symptom score. After typhoid diagnosis or 14 days without typhoid diagnosis all participants received a two week course of oral ciprofloxacin.

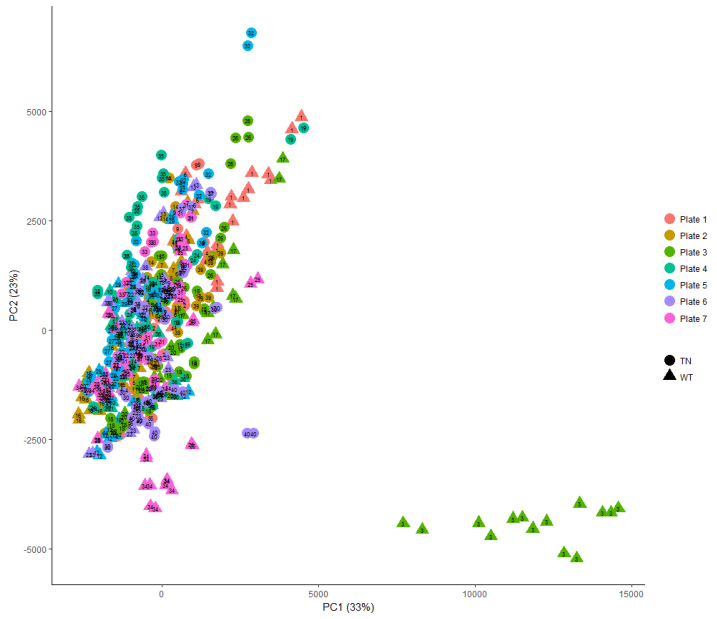
3.2.2 Plasma Cytokine Quantification

Plasma isolation was carried out by Oxford Vaccine Group research staff. Heparinised blood samples were collected and centrifuged at 3000rpm for 10 minutes at 4°C. The supernatant was transferred to a fresh tube before centrifuging a second time. The supernatant was then aliquoted and protease inhibitor added in a 1:40 dilution before storage at -80°C. Cytokine quantification was carried out for all 40 participants at various time points (Figure S3.1) by the The Human Immune Monitoring Center at Stanford University using a 62-plex Luminex assay custom-built by eBioscience. Samples were assayed in duplicate, with all samples for a particular participant assayed on one plate to allow comparison across time points. Seven plates were used in total.

3.2.3 Data Processing

Examination of cytokine concentrations by principal component analysis showed a notable batch effect by plate (Figure S3.2), while the median fluorescence intensity (MFI) did not, suggesting inconsistency in the standards used. Therefore MFI was chosen over concentration as a measure of plasma cytokine levels. One participant was excluded on the basis of an outlying MFI (Figure 3.1).

Figure 3.1: PC1 vs PC2 for a principal component analysis on the concentrations and raw median fluorescence intensities for each sample. Technical duplicates are shown as separate points. Samples are coloured by plate number, while the shape of each point represents challenge group. All of participant 3 samples were excluded.



Duplicates were then averaged and the MFIs quantile normalised (Figure S3.3). Where appropriate, samples were grouped into the time points D0 (baseline), D0.5 (12 hours after challenge) D1 (day 1), D2 (day 2), D4 (day 4), pre-TD (24-48 hours before typhoid diagnosis), TD (time of typhoid diagnosis), post-TD (12-24 hours after diagnosis), D10 (day 10 after challenge in non-diagnosed participants, acting as a comparator to TD in diagnosed participants) and D14.

Results from this study were correlated with existing data from two independent cohorts: a typhoid dose-escalation study and a Vi polysaccharide vaccine trial (see Chapter 2.2.1 and 2.2.4). Plasma was isolated as above. Samples were run in duplicate on MILLIPLEX MAP kits according to manufacturer's instructions. Duplicates were averaged and only samples which passed quality control or were run on the same plate as the matched baseline were included.

3.2.4 Validation

Plasma aliquots from a subset of participants in the typhoid toxin study (n = 37) and bicarbonate extension study (n = 9) at baseline and 12 hours post-challenge were re-run in duplicate using a MILLIPLEX MAP kit with nine analytes (EGF, TGFA, CXCL1, CD40L, IL1RA, IL2, IL6, IL8 and TNFA). As BDNF was incompatible with this panel, changes in BDNF were validated using an ELISA Kit (abcam ab212166) carried out according to manufacturer's instructions in duplicate. The BDNF ELISA was carried out for 21 participants in the typhoid toxin study and 8 unchallenged controls on plasma samples from baseline and 12 hours post-challenge/bicarbonate administration. Duplicates were averaged for both assays.

3.2.5 *In vitro* stimulation of leukocytes by *S.* Typhi Quailes strain

10ml heparinised blood from three healthy human volunteers was incubated at room temperature for 15 minutes in three volumes of red blood cell (RBC) lysis solution (Qiagen). White blood cells were centrifuged at 500G for 5 minutes, and washed with a further three

volumes of RBC lysis solution then three volumes autoMACS Running Buffer. Cells were resuspended in RPMI with 10% fetal bovine serum (FBS) at 10^6 cells/ml and seeded in a 24 well plate. A frozen stock of *S.* Typhi Quailes strain was thawed, washed twice with RPMI (plus 10% FBS) then diluted to the specified CFU/ml. Cells were stimulated either with *S.* Typhi at an MOI of 10 or 0.1, 100ng/ml *S.* Typhi LPS (L2387, Sigma-Aldrich) as a positive control, or RPMI as a negative control. After one hour 100µg/ml gentamycin was added to kill extracellular bacteria. After a further two, four or eight hours, cells were centrifuged at 500G for 5 minutes, and the supernatants filtered using 0.22µm cellulose acetate Spin-X centrifuge tube filters.

3.2.6 Individual *in vitro* stimulation of granulocytes, monocytes and lymphocytes by *S.* Typhi Quailes strain

To characterise cytokine secretion by different leukocyte subsets, 15ml blood was collected from each of three healthy donors in an EDTA vacutainer, and layered on to an equal volume of Polymorphprep™ (Alere Technologies) then centrifuged at 500G for 35 minutes at room temperature with the brake off. The granulocyte and PBMC bands were transferred to separate falcon tubes and topped up to 50ml with PBS. Cells were centrifuged at 250G for 10 minutes then resuspended in 5ml RBC lysis solution for 5 minutes. The tube was once more topped up to 50ml with PBS, centrifuged at 250G for 10 minutes, and the pellets resuspended in 4ml PBS for cell counting. CD14 microbeads (Miltenyi Biotec) were diluted 1 in 5 in autoMACs running buffer and PBMCs suspended in 300µl diluted beads. After 15 minutes at 4°C 6ml autoMACS running buffer was added, and the PBMCs centrifuged for 10 minutes at 250G. PBMCs were then resuspended in 500ul running buffer, and the $CD14^+$ fraction (monocytes) and $CD14^-$ fraction (lymphocytes) separated using an autoMACS Pro Separator. Viability was assessed using trypan blue and was high. Purity, as previously determined for this separation method by Helena-Thomaides Brears, is outlined in table 3.2.

Table 3.2: Purity of cell fractions following density gradient centrifugation and $CD14^+$ selection on the PBMC band	
Density Gradient Centrifugation	
Fraction	Purity
Granulocytes	69.4-80.6% (10.2-11.5% PBMCs)
PBMCs	88.6% (1.1% granulocytes)
$CD14^+$ selection on PBMCs	
Fraction	Purity
Monocytes	81.7% (1.97% lymphocytes)
Lymphocytes	85% (2.55% monocytes)

Monocytes, lymphocytes and granulocytes were counted using a haemocytometer. suspended in RPMI and seeded in duplicate (two wells per donor per cell type) in a 96 well plate at a density of 100,000 cells per well. Frozen stocks of 10^7 CFU/ml Quailes strain *S.* Typhi were thawed and washed twice with RPMI. Cells were inoculated with *S.* Typhi at an MOI of 10 or RPMI as a negative control. After 50 minutes, gentamycin was added to a final concentration of 200ug/ml. Four hours post-inoculation, the plate was centrifuged at 400G for 4 minutes, and the supernatants filtered using 0.22μm cellulose acetate Spin-X centrifuge tube filters.

3.2.7 *In vitro* stimulation of leukocytes by different *Salmonella* strains

Frozen glycerol stocks of *S.* Typhi Quailes strain, isogenic toxin-negative *S.* Typhi SB6000 strain, *S.* Paratyphi A NVGH308 strain, attenuated *S.* Typhi Ty2 strain derivatives CVD 908 and CVD 909 (Tacket and Levine 2007), *S.* Typhimurium LT2 strain, and constitutive Vi^+ *S.* Typhimurium were used to inoculate 5ml LB broth and incubated at 37°C overnight. Two further cultures of *S.* Typhi Quailes strain took place under high (0.3M) and low (9mM) NaCl conditions. Overnight cultures were diluted to a concentration of 5 x 10^8 CFU/ml, added to an equal volume of 50% glycerol in H_2O, and frozen. 5ml heparinised blood from one healthy human volunteer was incubated at room temperature for 15 minutes in three volumes of RBC lysis solution (Qiagen). As in 3.2.5, white blood cells were centrifuged at 500G for 5 minutes, and washed with a further three volumes of RBC lysis solution then three volumes autoMACS Running Buffer. Cells were resuspended in RPMI with 10% FBS and seeded in a 96 well plate at a density of 100,000 cells per well. Bacterial stocks were simultaneously thawed, with one vial

of *S.* Typhimurium LT2 strain heat-killed by a 20 minute incubation at 65°C. Bacteria were washed twice with RPMI, and cells inoculated at an MOI of 10 in duplicate or with RPMI as a negative control. At 2 and 4 hours post-infection cells were centrifuged at 400G for 4 minutes, and the supernatants filtered using 0.22μm cellulose acetate Spin-X centrifuge tube filters.

3.2.8 Supernatant cytokine quantification

Cytokine levels were quantified in the supernatants from 3.2.5, 3.2.6 and 3.2.7 using a MILLIPLEX MAP kit with nine analytes (EGF, TGFA, CXCL1, CD40L, IL1RA, IL2, IL6, IL8 and TNFA) as described in 3.2.4. The MILLIPLEX MAP kit was also used to quantify cytokines in the supernatants intestinal organoids (Lees et al. 2019) stimulated with *S.* Typhi Quailes strain, carried out by Emily Lees. The cells from each donor were stimulated in triplicate, and cytokine quantification carried out on each stimulation in duplicate. Cytokine measurements from each stimulation were averaged. A BDNF ELISA (abcam ab212166) was carried out as described in 3.2.4 for *S.* Typhi Quailes strain stimulated leukocytes (3.2.5).

3.2.9 Statistical analysis

All statistical analysis was carried out using the R Statistical Package. Significance testing for changes in 62-plex eBioscience assay cytokine profile compared with baseline was performed using linear modelling in limma, as this approach allows inclusion of covariates, estimates within-participant correlation, and improves power through use of an empirical Bayes method to moderate the standard errors of the estimated $\log_2$(fold changes). Plate, dose and sex were modelled as covariates for all comparisons, and challenge strain group as a covariate where inter-group comparisons were not being made. As a large number of cytokines were being tested, P values were corrected for multiple testing using the Benjamin-Hochberg correction.

Limma was also used to assess the differences in time course between those who were and were not diagnosed with typhoid fever, and between challenge strain groups. A cubic spline

curve was fit to each group, and the difference between the time courses of each group assessed using a moderated F-test for each cytokine with 3 degrees of freedom.

Two methods of unsupervised clustering were carried out: Euclidian distance and principal component analysis in order to identify factors (timepoint, outcome, group, batch) with the greatest overall effect on cytokine profile.

A Bland-Altman analysis was used to assess the agreement between multiplex cytokine assays carried out on different cohorts and Luminex platforms, as unlike a correlation analysis it can identify proportional bias.

A Mann–Whitney–Wilcoxon test was used to compare the 12 hour plasma cytokine response between challenged participants and unchallenged controls, as samples were unpaired and the distribution was positively skewed, and also to compare the fold change in acute disease cytokines between vaccination groups.

A two-sample t-test was used where a small (2-6) number of replicates would have otherwise limited the ability to distinguish between a large and small magnitude of difference in the means between groups: to compare cytokine secretion profiles between stimulated and unstimulated leukocytes, between stimulated and unstimulated intestinal organoids, between granulocytes, monocytes and lymphocytes, and between leukocytes stimulated with different *Salmonella* serovars. A one-sample t-test was used to assess whether cytokine secretion by each of the cell subsets was greater than zero.

Pearson correlation analyses were carried out to evaluate linear relationships between continuous variables, including between principal components and time elapsed since challenge; between $\log_2$(Fold change in MFI) in cytokines and temperature, symptom score and C-reactive protein; and between $\log_2$(Fold change in MFI) in cytokines from the same

participant carried out on different Luminex platforms. P-values indicate the probability of achieving this level of correlation if the variables were unrelated.

3.3 Results

3.3.1 Samples weakly cluster by time point but not by outcome or group

Plasma cytokines were longitudinally profiled using a 62-plex eBioscience Luminex assay (kinetics summarised in Figure 3.2). Changes in cytokines between baseline and each of the post-challenge time points appear to cluster weakly by time point (Figures 3.3 and 3.4a), with principal component 2 correlating significantly with time elapsed since challenge ($\rho = 0.45$, $p = 1.6 \times 10^{-15}$, Figure 3.2b and 3.2c). Given that the samples for each participant were all run on one plate, we believe this is due to biological changes rather than technical batches. There is little clustering at each individual time point by outcome or challenge strain (Figure S3.4 and S4.5).

Figure 3.2: Log_2(fold change) in each cytokine for plasma samples from the typhoid toxin challenge study were longitudinally profiled over the course of infection using a 62-plex Luminex assay. Each point represents one sample, with samples corresponding to non-diagnosed participants shown in blue and those corresponding to diagnosed participants shown in pink. Time is shown as day relative to challenge. Loess curves are shown with shading indicating 95% confidence intervals.



Figure 3.3: Log_2(fold change) in MFI for each sample relative to baseline. Samples and plasma cytokines are both clustered by Euclidean distance. Bars between the heat map and column dendrogram indicate time point (lighter colours = early time points, darker colours = later time points), outcome (maroon = diagnosed, blue = non-diagnosed), challenge group (green = WT, yellow = TN) and plate number.

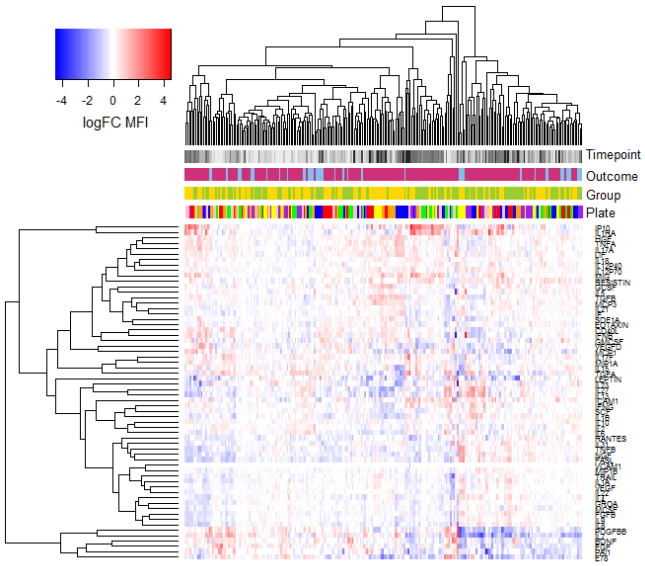
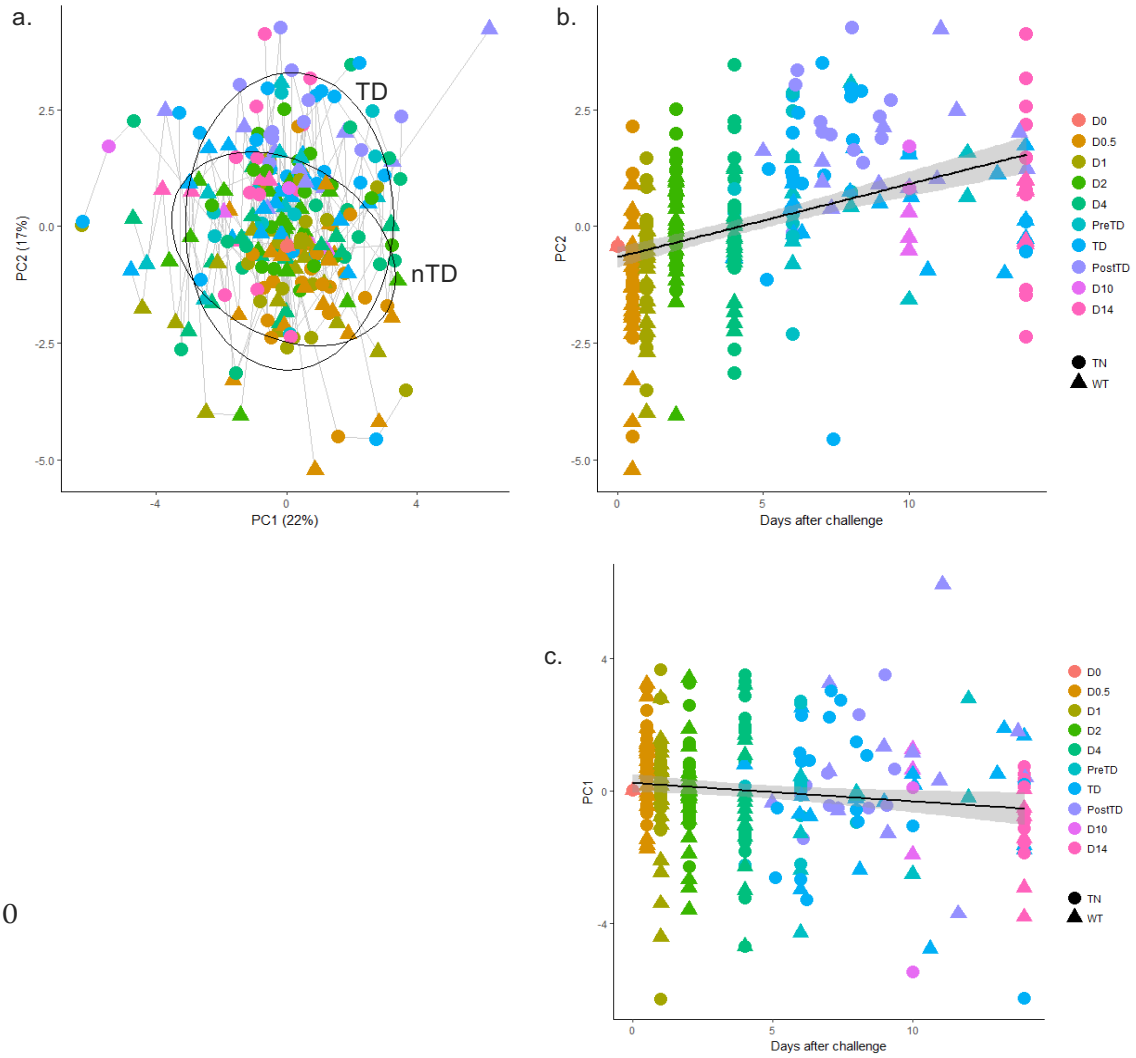


Figure 3.4a: First two principal components (PC1 and PC2), each point representing a sample. Points are coloured by time point, while shape corresponds to challenge group and ellipses the 95% confidence intervals by outcome of challenge (TD = developed typhoid, nTD = remained healthy). Samples from the same participant are linked by a grey line.
b: Correlation between principal component 2 (PC2) and time elapsed since challenge. Points are coloured by time point, while shape corresponds to challenge group. $\rho = 0.45$, $p = 1.6 \times 10^{-15}$
c: Correlation between PC1 and time elapsed since challenge. $\rho = -0.14$, $p = 0.017$



3.3.2 Plasma cytokines are most perturbed during acute disease and at 12 hours after challenge

Linear modelling was used to assess whether cytokines were increased or decreased relative to baseline at each time point (Figure 3.5). In those who went on to develop clinical disease, the time points with the greatest number of significantly changed cytokines, defined as having an FDR < 0.05, were Post-TD (12-24 hours after diagnosis), TD (time of diagnosis) and D0.5 (12 hours after challenge, the estimated time of primary bacteraemia). Separate curves were fit to the time courses of those who did and did not develop typhoid in order to identify cytokines which had different overall patterns between the two outcome groups (Figure 3.6), of which IL1RA and IP10 were the most significant (FDR < 0.05, moderated F-test).

Figure 3.5: Amongst participants who developed enteric fever, linear modelling was used to assess up- and down-regulation of cytokines relative to baseline. Each line corresponds to a time point. Time points where greater numbers of cytokines are significant at each p value threshold are considered more perturbed from baseline: for example amongst participants who were diagnosed, at Post-TD p < 0.05 for 25 cytokines and at Day 2 p < 0.05 for one cytokine

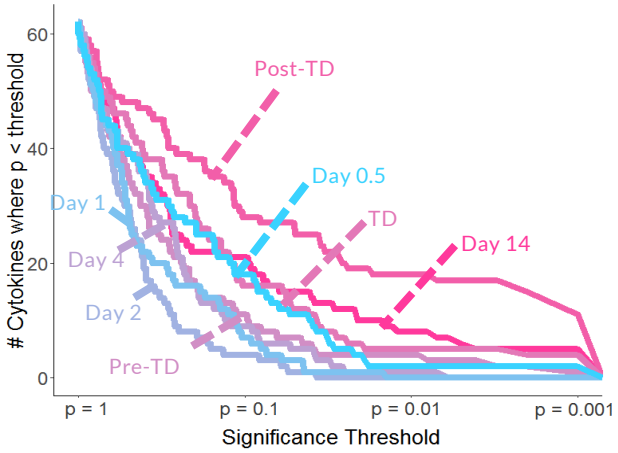
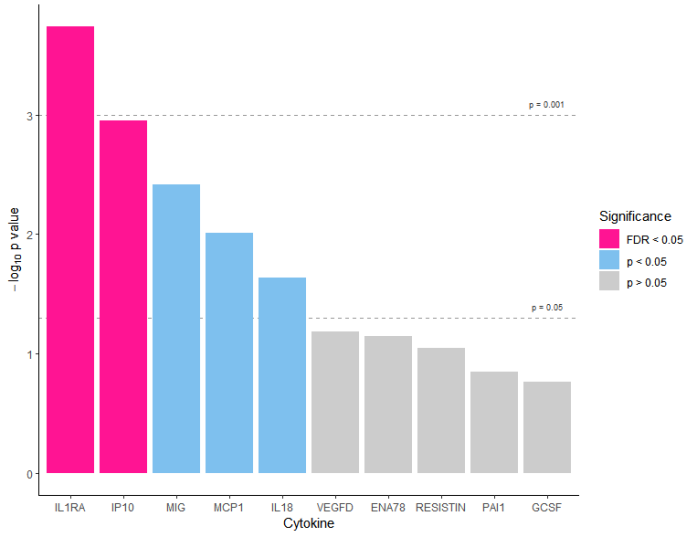


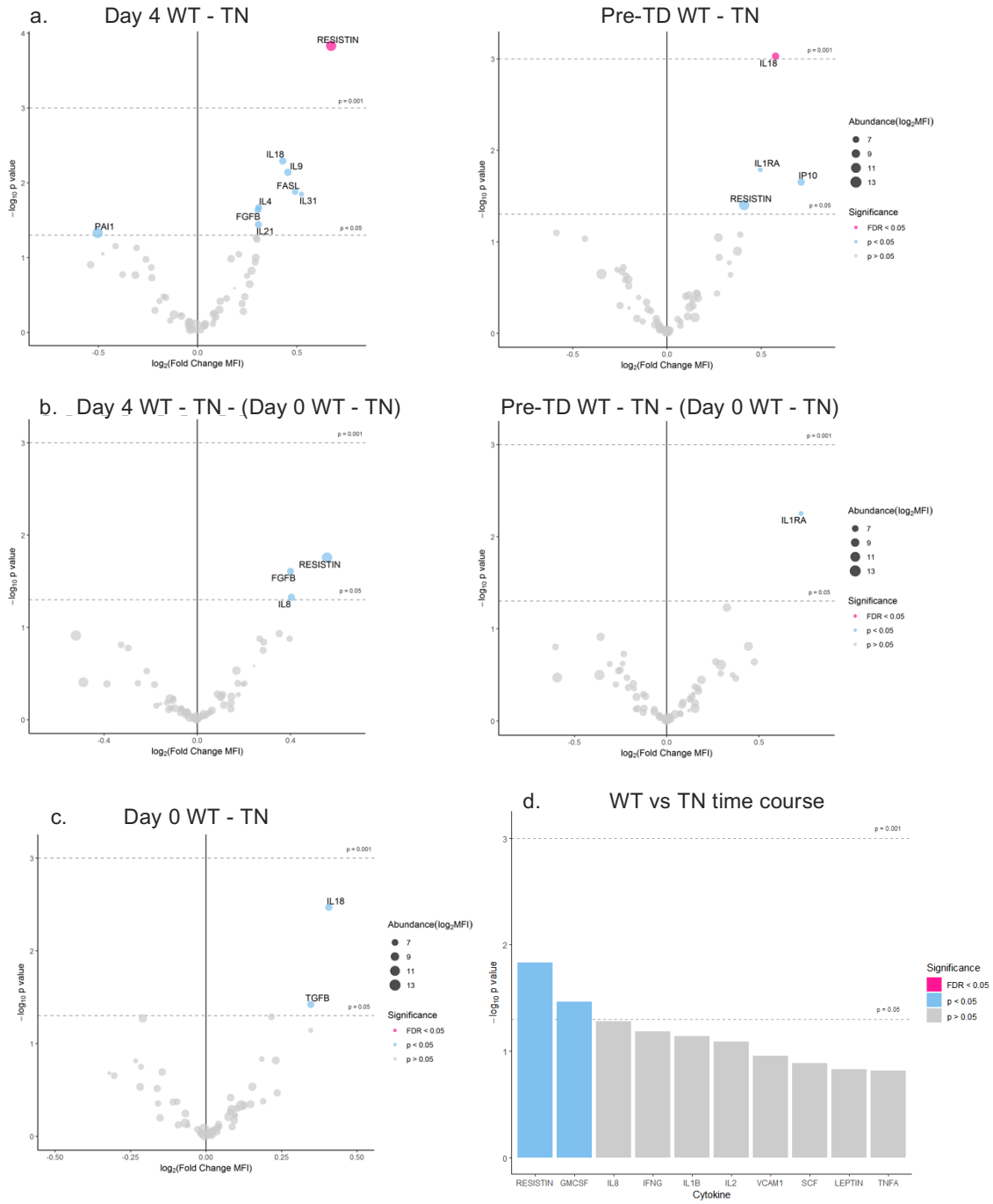
Figure 3.6: The -$\log_{10}$ p values of a moderated F-test for each cytokine with 3 degrees of freedom, assessing the overall difference between participants who were and were not diagnosed with typhoid. Those which were significantly different between the two time courses after adjusting for multiple testing are shown in pink, and before adjustment in blue.



3.3.3 Toxin-negative Quailes strain *S.* Typhi elicits a similar cytokine profile to challenge with wild-type *S.* Typhi

Among participants diagnosed with typhoid, RESISTIN and IL18 were significantly higher in wild-type group at day 4 and Pre-TD respectively compared with those challenged with the typhoid toxin-negative *S.* Typhi strain (FDR = 0.009 and 0.03 respectively, linear modelling; Figure 3.7a). However, as IL18 is slightly higher in the wild-type group at baseline (Figure 3.7c, p = 0.003, FDR = 0.2), this difference disappeared when adjusting for baseline levels (Figure 3.7b). RESISTIN however was still slightly higher in the wild-type group at Day 4 (p = 0.02, FDR = 0.5). Comparing the overall time courses of each cytokine between the two groups, RESISTIN was the most different, although this did not reach significance after multiple testing adjustment (p = 0.01, FDR = 0.8, Figure 3.7d).

Figure 3.7: Volcano plots showing differences for each time point between the wild type and toxin negative group. Cytokines which were significant after adjusting for multiple testing using the Benjamin-Hochberg correction are shown in pink, and those significant before adjustment in blue. The size of the points reflects the average abundance (log_2MFI) of the cytokine.



3.3.4 BDNF and EGF are significantly raised 12 hours post challenge

As 12 hours post-challenge is the timepoint with the highest cytokine perturbation prior to development of acute disease, we next examined differences in cytokine levels at this timepoint relative to baseline. Before adjusting for multiple testing 9/62 cytokines are upregulated 12 hours after challenge and 2/62 downregulated ($p < 0.05$, linear modelling), while after adjustment 2/62 are upregulated (FDR < 0.05; Figure 3.8). The direction and magnitude of change is similar between those who did and did not develop disease (Figure 3.9). Network analysis was carried out to assess relationships between cytokines that were up-regulated before adjustment for multiple testing. Two networks were identified: an EGF/TGFB network, connected by EGFR, ERBB2 and ERRB4, and an IL2/IL6/IL15 network, connected by IL2RG and IL2RB (Figure 3.10).

Figure 3.8: Volcano plot showing significantly up and downregulated cytokines 12 hours after challenge, as assessed by linear modelling. Cytokines which were significant after adjusting for multiple testing (FDR < 0.05) using the Benjamin-Hochberg correction are shown in pink, and those significant before adjustment ($p < 0.05$) in blue. The size of the points reflects the average abundance (log_2MFI) of the cytokine.



Figure 3.9: Distribution of logFC MFIs at 12 hours post-challenge relative to baseline for cytokines with p < 0.05, stratified by outcome of challenge. Each point represents one participant.

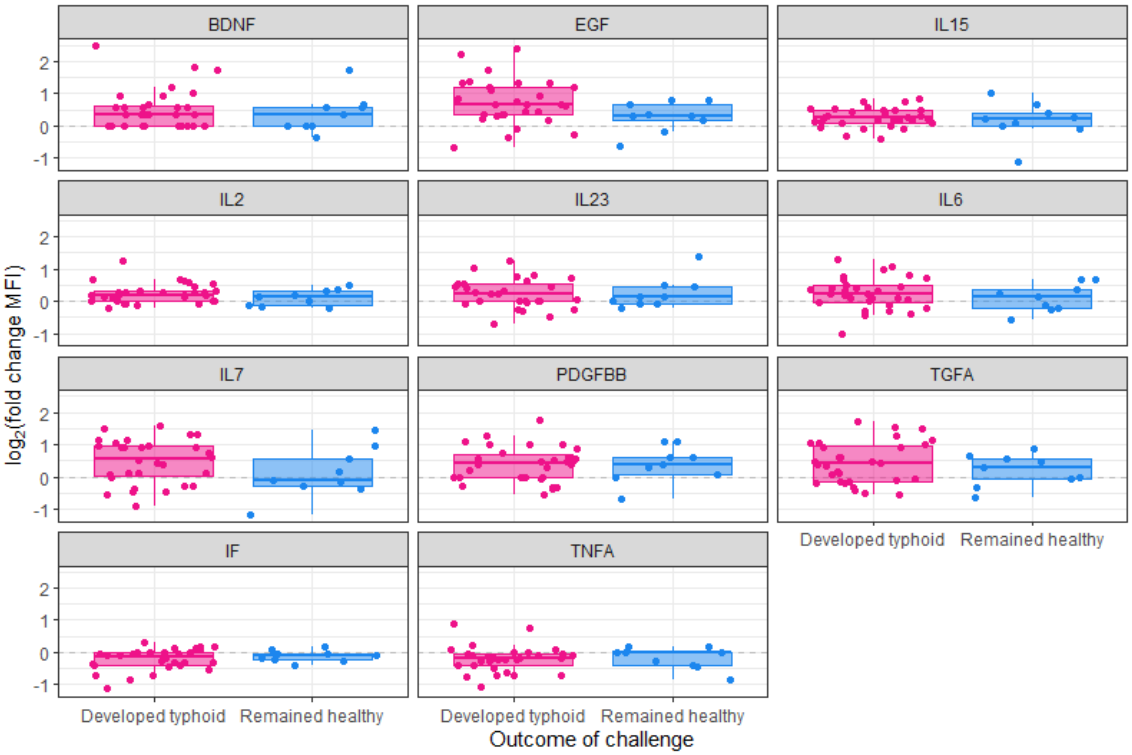
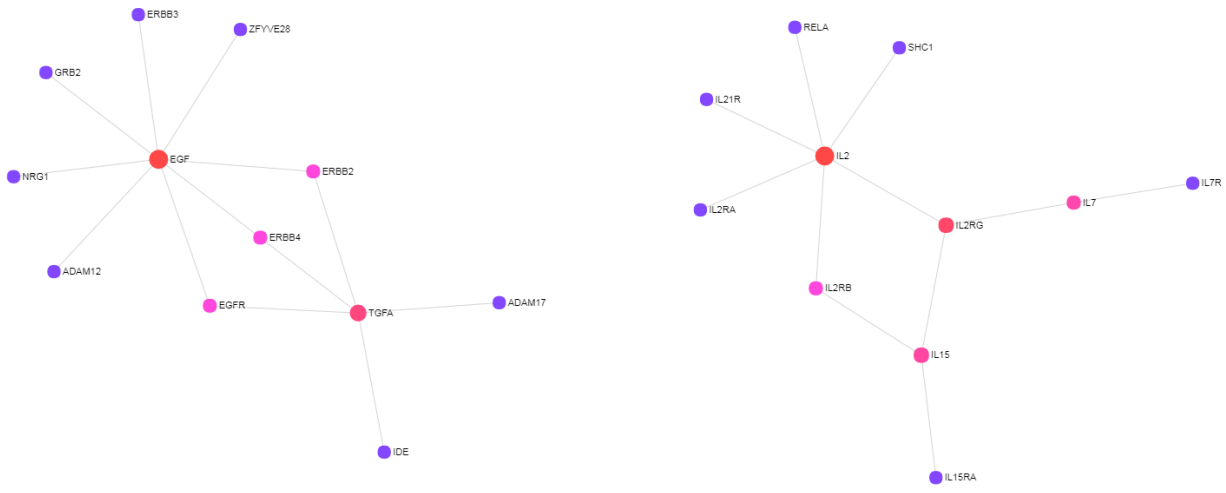


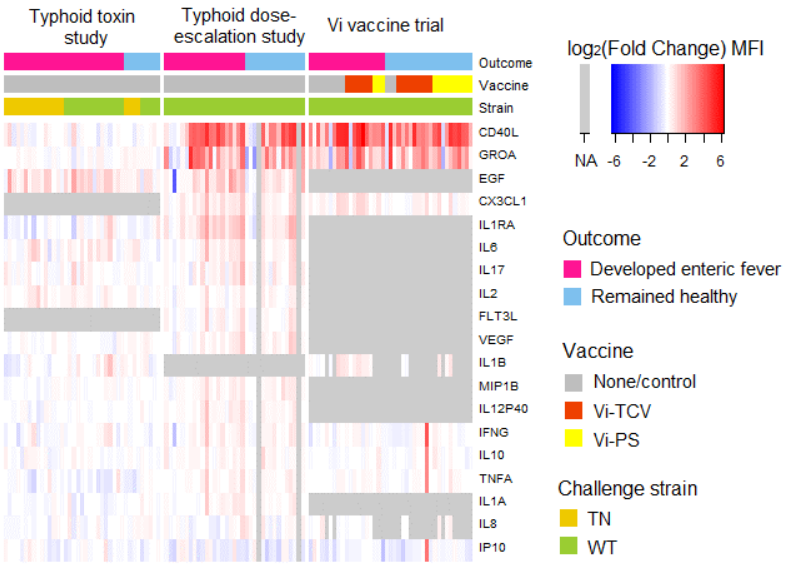
Figure 3.10: NetworkAnalyst networks showing the interactions between differentially regulated cytokines 12 hours after challenge and proteins according to STRING Interactome (confidence score > 900 confidence and requiring experimental evidence). Proteins with the fewest interactions are shown in purple, and with the greatest number of interactions in red.



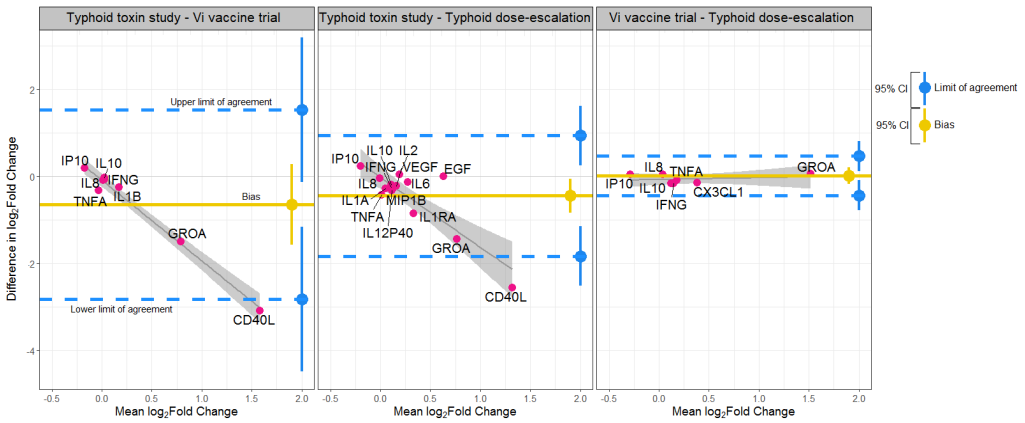
Given the inter-manufacturer variability between multiplex cytokine assays, results were compared with those from two independent cohorts (the typhoid dose-escalation study and Vi vaccine trial) where a smaller panel of plasma cytokines were profiled using a Millipore rather than an eBioscience Luminex kit (Figure 3.11a). A Bland-Altman analysis was carried out on the average $\log_2$(Fold Change MFI)s at 12 hours post-challenge for each study (Figure 3.11b). Making the assumption that biological responses to challenge were likely to be similar in the typhoid toxin and typhoid dose-escalation studies as all participants were un-vaccinated, there appears to be a negative bias which increases linearly with $\log_2$(Fold Change), suggesting that on average the eBioscience assay is less sensitive to changes in plasma cytokines. The agreement between the two studies carried out using the Millipore kit however was excellent.

Figure 3.11a: Heat map summarising the change in plasma cytokine levels between baseline and 12 hours post-challenge for each participant in the typhoid toxin, typhoid dose escalation, and Vi vaccine studies. b. Bland-Altman plots showing the difference in the log_2(Fold change MFI) for each cytokine between studies, and how this relates to the mean log_2(Fold change MFI) at 12 hours post challenge. A regression line with 95% confidence intervals is shown in grey. The bias is shown in yellow, with 95% confidence intervals illustrated by a bar. A confidence interval crossing y=0 indicates no significant bias. The upper and lower limits of agreement and corresponding 95% confidence interval are shown in blue.

a.



b.



To better assess inter-manufacturer and methodological differences, baseline and 12 hours samples from the typhoid toxin study were re-run using a Millipore kit to quantify EGF,TGFA, CXCL1, CD40L, IL1RA, IL2, IL6, IL8 and TNFA, and using an ELISA to quantify BDNF. Plasma from negative control participants who received bicarbonate only were run for comparison. There was a significant rise in EGF and BDNF relative to unchallenged controls (p = 0.005 and 0.004 respectively; Mann–Whitney-Wilcoxon test), but unlike the eBioscience assay, a rise in

secreted CD40L and GRO-α was also detected (p = 0.05 and 0.002 respectively relative to unchallenged controls; Figure 3.12). Correlation between the eBioscience and Millipore kits was positive and significant for CD40L, EGF, IL1RA, TGFA and IL2 ($p < 0.05$, $\rho > 0.3$; Pearson correlation) and positive but non-significant for IL6 (p = 0.4, ρ = 0.1; Figure 3.13). Correlation was also significant between the eBioscience kit and ELISA for BDNF (ρ = 0.6, $p = 7 \times 10^{-6}$). However, there was a negative correlation between the two multiplex kits for TNFA, IL8 and GRO-α.

Figure 3.12: Log_2(fold change in MFI) of each cytokine in the plasma relative to baseline, either 12 hours post-challenge or 12 hours after ingestion of bicarbonate as a negative control, as measured by Millipore Luminex kit or ELISA. Each point represents one participant. P values for a Mann–Whitney–Wilcoxon test are shown.

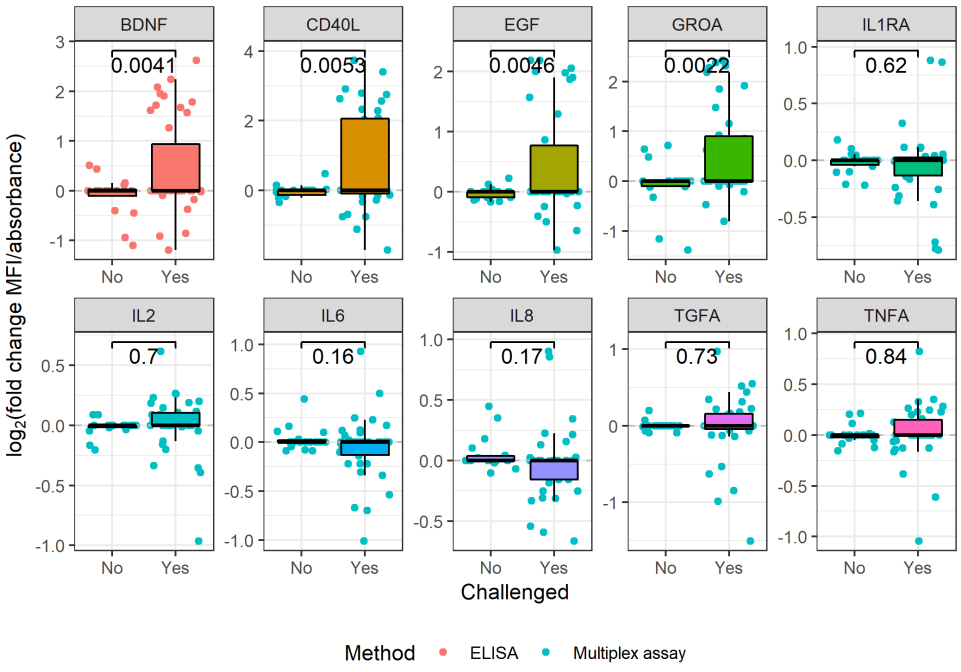
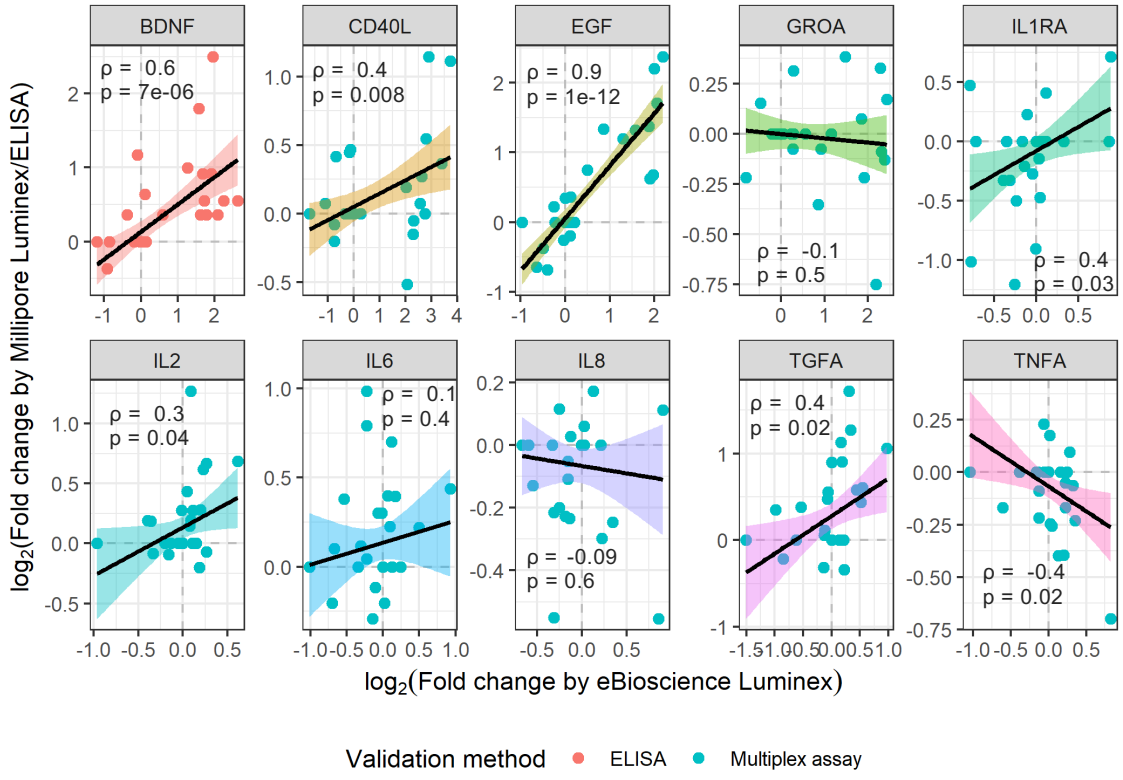


Figure 3.13: Correlation in $\log_2$(fold change MFI) at 12 hours post-challenge relative to baseline for each participant, measured by either eBioscience Luminex, Millipore Luminex or ELISA. A linear regression line is shown with shading indicating 95% confidence intervals. Pearson's product-moment correlation coefficient (ρ) and the p-value for a test of whether ρ is different from zero are shown above each scatterplot



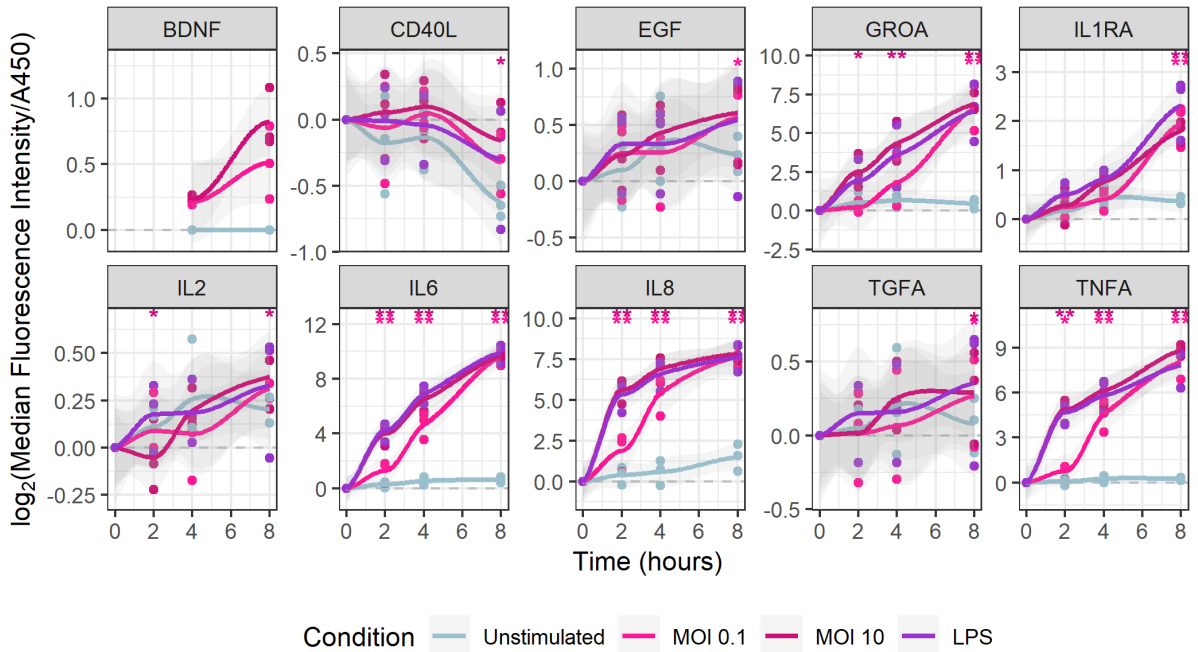
3.3.5 Early response cytokines are produced by white blood cells in response to stimulation with *S.* Typhi

In order to identify potential sources of the plasma cytokine signature observed early after challenge, white blood cells were stimulated with *S.* Typhi (Quailes strain) to assess their ability to produce 'early response cytokines' upon infection. Cytokines were chosen based on early changes observed using the Millipore kits (EGF, CXCL1, CD40L, IL1RA), early changes observed using the eBioscience kit (EGF, BDNF, TGFA, IL2, IL6, and TNFA) or changes observed *in vitro* in previous literature (IL8) (Nickerson et al. 2018; Salerno-goncalves et al. 2019; Winter et al. 2008). BDNF was again quantified by ELISA and the other cytokines by Millipore Luminex kit, on the basis of inter-study reproducibility having been high. An MOI of 10 was chosen on the

basis of this having elicited robust IL-8 secretion from leukocytes in a preliminary titration experiment (Figure S3.6), and on previous literature (see Table 3.1). However as this is approximately 10^8 fold the MOI of *in vivo S.* Typhi bacteraemia, a somewhat lower MOI of 0.1 was also tested.

While the fold change in cytokine concentration was most marked for CXCL1, IL6, IL8 and TNFA, all cytokines other than CD40L profiled by the Millipore Luminex kit were increased in the supernatant of stimulated white blood cells by 8 hours post-inoculation relative to an unstimulated control (Figure 3.14, $p < 0.1$, one-sample t-test). BDNF was consistently raised in the supernatant for each donor following stimulation, although the difference between stimulated and unstimulated conditions was non-significant.

Figure 3.14: Cytokine production by leukocytes from three donors stimulated with *S.* Typhi at an MOI of 0.1, *S.* Typhi at an MOI of 10, LPS or unstimulated. Heparinised blood was collected from three healthy volunteers, and white blood cells isolated by red blood cell lysis and washing. A frozen stock of *S.* Typhi Quailes strain was thawed, washed and added to white blood cells at an MOI of 0.1 or 10. Cells were stimulated with 100ng/ml *S.* Typhi LPS as a positive control and RPMI as a negative control. After one hour 100µg/ml gentamycin was added to kill extracellular bacteria. After a further two, four or eight hours, supernatants were harvested for cytokine quantification using a Merck Millipore kit. A loess curve is fitted for each condition with 95% confidence intervals shaded in grey. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.1$ for a t test comparing the stimulated to the unstimulated condition at each time point.



To investigate in more detail the ability of different cell populations to produce these cytokines, cytokine secretion from granulocytes, monocytes, lymphocytes (one donor, 4 hours post-inoculation, Figure 3.15) and intestinal organoids (three donors, 1.5 and 3 hours post-inoculation, Figure 3.16) stimulated separately with Quailes strain *S.* Typhi was profiled using the Millipore Luminex kit. IL8, TNFA, IL6 and GRO-α were produced by all three leukocyte subsets (one sample t-test, $p < 0.1$), with TNFA and IL6 produced to the greatest extent by monocytes, IL8 by granulocytes, and GRO-α by both granulocytes and monocytes (two sample t-test, $p < 0.1$). Of the leukocyte subsets, only granulocytes secreted CD40L, EGF, IL2 and IL1RA. Cytokine secretion by intestinal organoids was less marked, with organoids from one donor producing GRO-α and TGFA, and organoids from another producing IL1RA in response to infection.

Figure 3.15: Cytokine production by granulocytes, monocytes and lymphocytes from three donors stimulated with *S.* Typhi at an MOI of 10 after 4 hours, relative to an unstimulated control. Granulocytes and PBMCs were separated by density gradient centrifugation. An autoMACS Pro Separator was then used to separate $CD14^+$ monocytes from $CD14^-$ lymphocytes in the PBMC fraction. For each donor each leukocyte fraction was seeded in duplicate. A frozen stock of *S.* Typhi Quailes strain was thawed, washed and added to white blood cells at an MOI of 10. RPMI was used as a negative control. After 50 minutes, gentamycin was added to a final concentration of 200μg/ml to kill extracellular bacteria. The supernatant was harvested four hours post-inoculation for cytokine quantification using a Merck Millipore Luminex kit. Black asterisks indicate significance of differences between cell types (two sample t-test) and blue asterisks whether the change in cytokine production was significantly different from zero (one sample t-test). *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.1$ for a t test comparing the stimulated to the unstimulated condition at each time point.

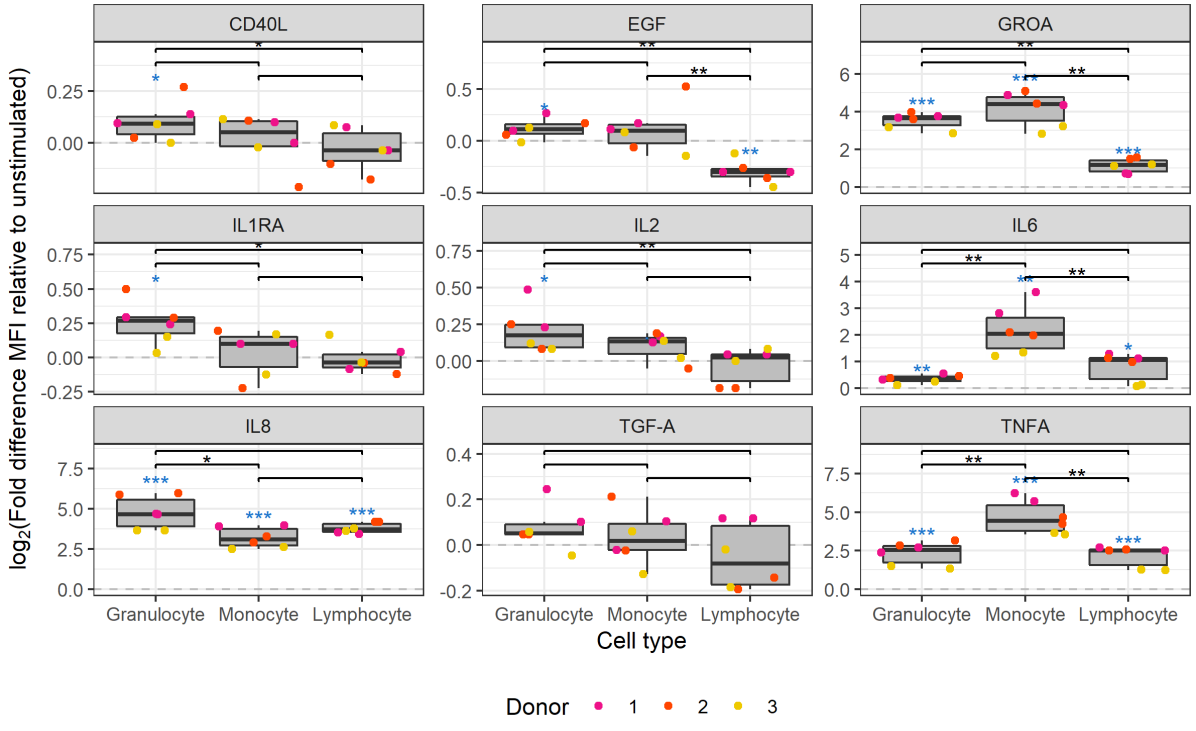
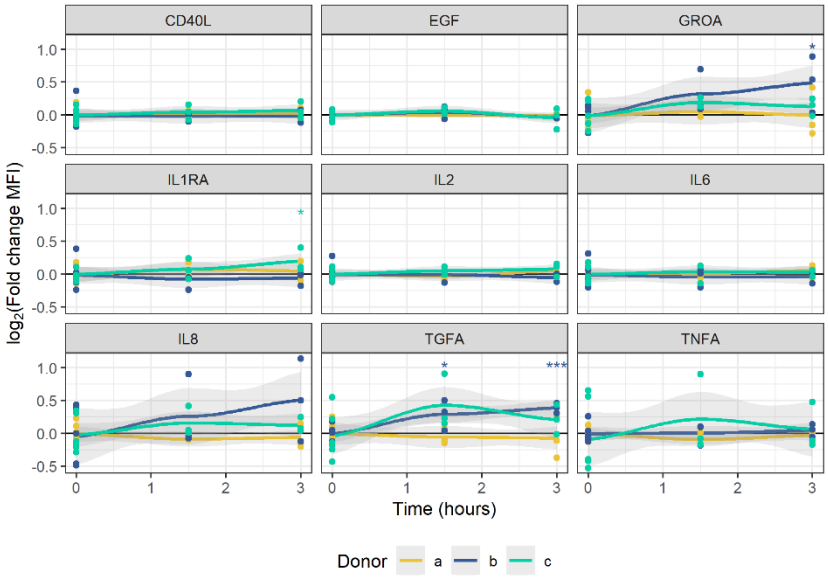
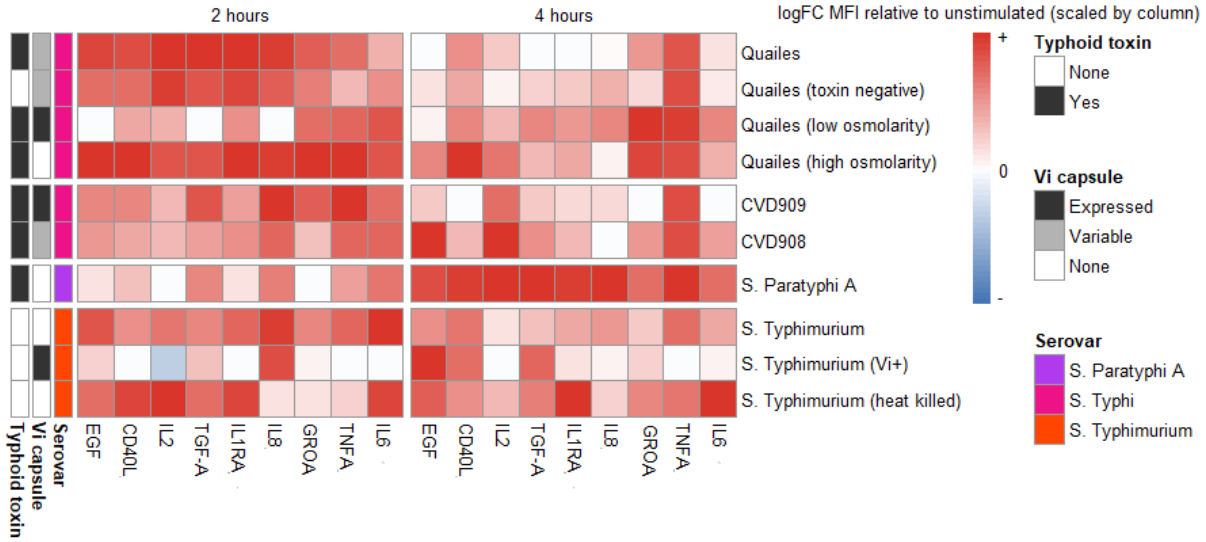


Figure 3.16: Changes in supernatant cytokine levels for iPSC-derived intestinal organoids from three donors stimulated with *S.* Typhi, each carried out in triplicate. Three healthy donor iPSC lines differentiated into hindgut and embedded in a basement membrane matrix were microinjected with Quailes strain *S.* Typhi. Supernatants were harvested after 1.5 or 3 hours and cytokine quantified using a Merck Millipore Luminex kit. A loess curve is fitted for each condition with 95% confidence intervals shaded in grey. *** = p < 0.001, ** = p < 0.01, * = p < 0.1 for a t test comparing the stimulated to the unstimulated condition at each time point.



Finally, white blood cells were stimulated using different strains of *Salmonella* to assess whether the Vi capsule and typhoid toxin modulate cytokine secretion in this model, and whether living bacteria are necessary to elicit a cytokine response (Figure 3.17). No differences were observed between stimulation with wild-type *S.* Typhi Quailes strain and the toxin-negative strain. At four hours post-stimulation, more IL1RA was produced in response to *S.* Paratyphi A and *S.* Typhimurium than *S.* Typhi Quailes strain (p = 0.03 and 0.01 respectively; two sample t-test). At this time point, *S.* Paratyphi A induced more IL6 than *S.* Typhimurium (p = 0.03) and more IL8 than *S.* Typhi (p = 0.01). Stimulation with *S.* Typhimurium expressing the Vi capsule resulted in lower secretion of GRO-α (p = 0.04 at 2 hours) and IL6 (p = 0.03 at 2 hours, p = 0.009 at 4 hours) than wild-type *S.* Typhimurium. However, constitutive Vi^+ CVD 909 elicited more secretion of GRO-α 2 hours post-stimulation than parent strain CVD 908 (p = 0.02). At 2 hours after inoculation, live *S.* Typhimurium induced in higher levels of GRO-α and IL8 than heat-killed *S.* Typhimurium (p = 0.05 and p = 0.01 respectively), but at 4 hours heat-killed *S.* Typhimurium resulted in greater secretion of IL6 (p = 0.01).

Figure 3.17: Cytokine secretion (log_2(fold change MFI relative to unstimulated cells), scaled by column) by leukocytes 2 and 4 hours post-stimulation with a variety of *Salmonella* strains, the properties of which are indicated in the side bar. Frozen glycerol stocks of S. Typhi Quailes strain, isogenic toxin-negative S. Typhi SB6000 strain, S. Paratyphi A NVGH308 strain, attenuated S. Typhi Ty2 strain derivatives CVD 908 and CVD 909, S. Typhimurium LT2 strain, and constitutive Vi+ S. Typhimurium were used to inoculate 5ml LB broth and incubated at 37°C overnight. Two further cultures of S. Typhi Quailes strain took place under high (0.3M) and low (9mM) NaCl conditions. Overnight cultures were diluted to a concentration of 5 x 10^8 CFU/ml, added to an equal volume of 50% glycerol in H2O, and frozen. Heparinised blood was collected from one healthy volunteer, and white blood cells isolated by red blood cell lysis and washing.. Bacterial stocks were simultaneously thawed, with one vial of S. Typhimurium LT2 strain heat-killed by a 20 minute incubation at 65°C. Bacteria were washed with RPMI, and cells inoculated at an MOI of 10 in duplicate or with RPMI as a negative control. At 2 and 4 hours post-infection supernatants were harvested for quantification using a quantified using a Merck Millipore Luminex kit



3.3.6 Changes in plasma cytokine profile during acute disease

Accounting for multiple testing, 2/62, 5/62, 18/62 and 7/62 cytokines were significantly changed relative to baseline at the Pre-TD, TD, Post-TD and D14 time points respectively (FDR < 0.05, linear modelling; Figure 3.18). At time of diagnosis, the cytokines with the greatest increase were IP10, IL1RA and MIG ($p = 1 \times 10^{-8}$, $p = 8 \times 10^{-8}$, $p = 8 \times 10^{-8}$ respectively, Figure 3.19).

Figure 3.18: Volcano plots showing significantly changed cytokines around the time of acute disease relative to baseline, as assessed using linear modelling. Cytokines which were significant after adjusting for multiple testing using the Benjamin-Hochberg correction are shown in pink, and those significant before adjustment in blue. The size of the points reflects the average abundance (log_2MFI) of the cytokine.

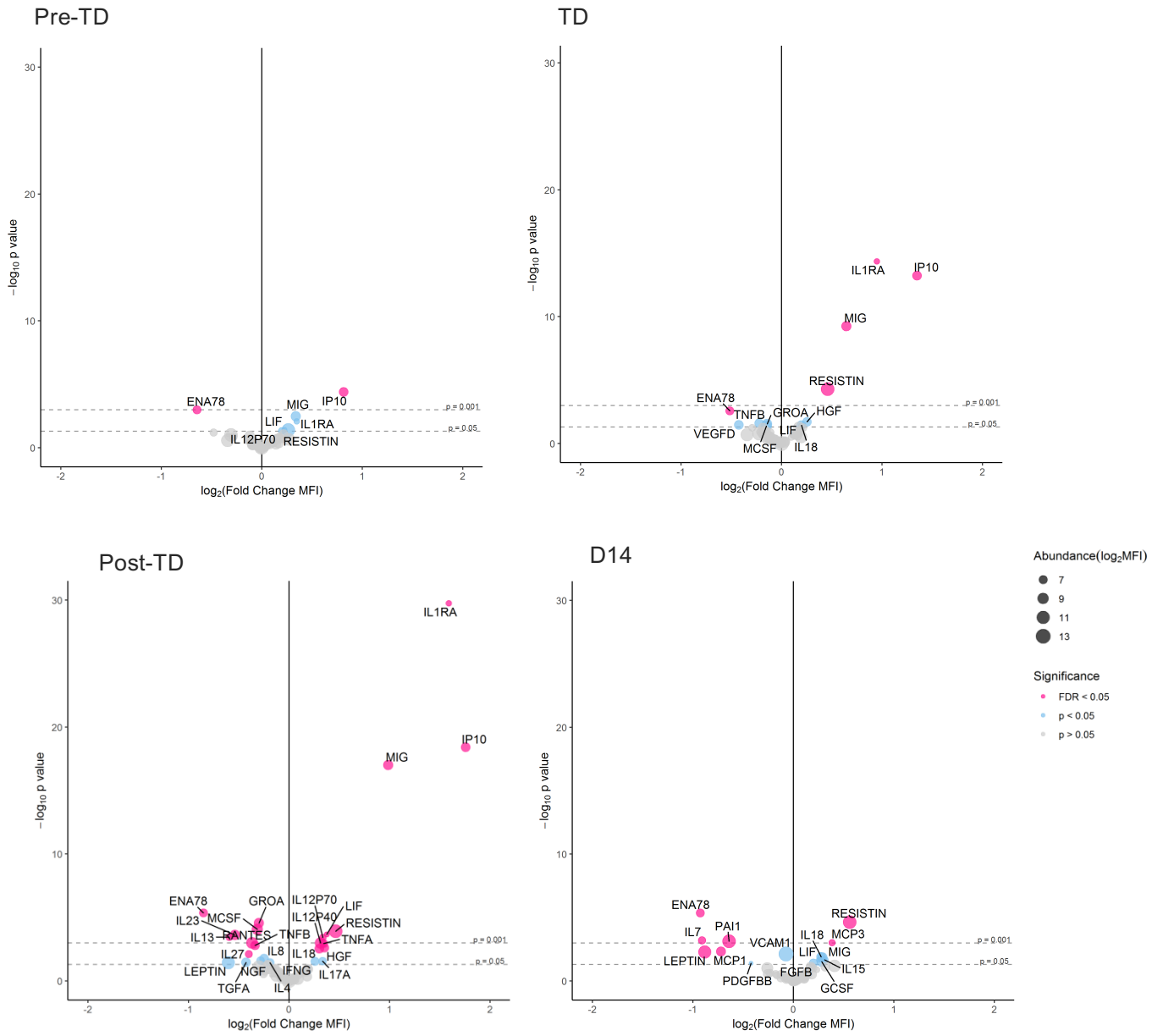
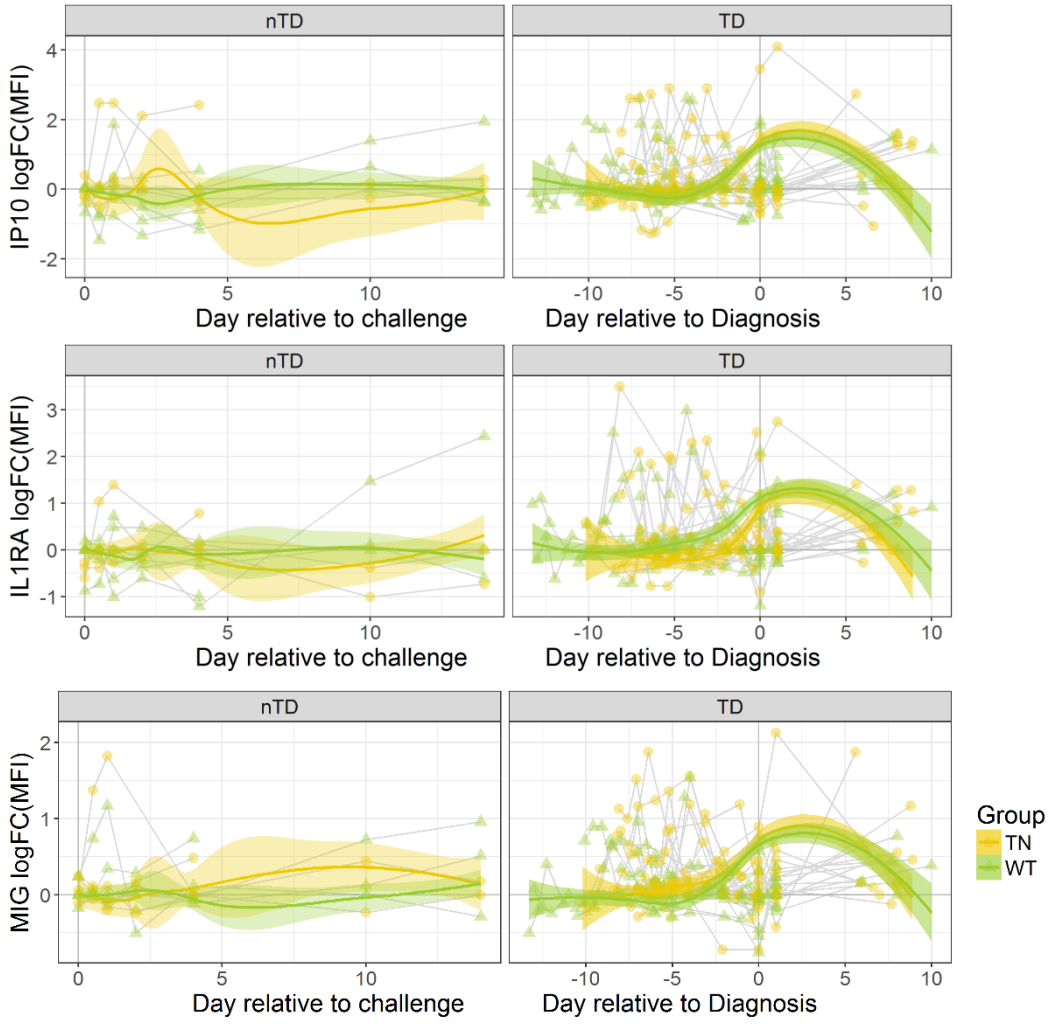


Figure 3.19: Kinetics of cytokines differentially regulated during acute disease. Each point represents one sample, with samples corresponding to non-diagnosed participants shown in the left panel for each cytokine and those corresponding to diagnosed participants on the right. Time is shown as day relative to challenge for non-diagnosed participants, and day relative to diagnosis for diagnosed participants. Points are coloured by challenge group. Loess curves are shown with shading indicating 95% confidence intervals.



3.3.7 IL1RA, IP10 and MIG are associated with higher fever and inflammation

As the incidence of participants meeting pre-specified criteria for severe disease was somewhat higher in the toxin-negative challenge strain group versus the wild-type group (4/19 and 1/21 respectively), we were interested in investigating whether any of the cytokines somewhat raised in the toxin-negative challenge strain group were related to disease severity.

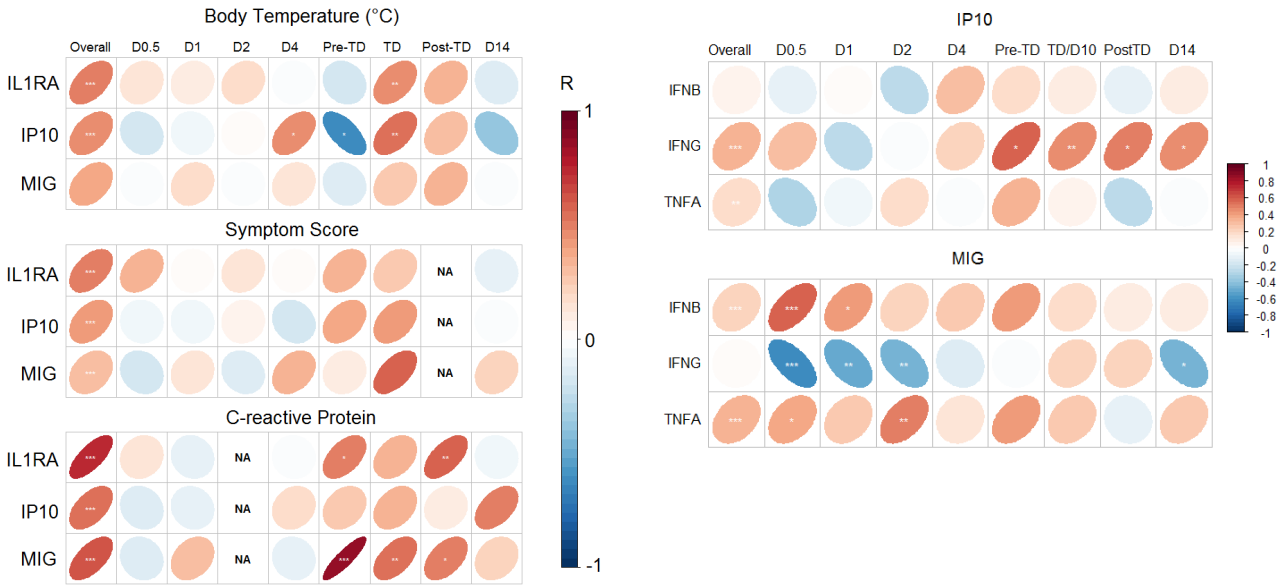
In the field, severe typhoid fever is generally defined by complications such as intestinal perforation, myocarditis, shock and death (Dunstan et al. 2005; Parry et al. 2014). While in human challenge studies volunteers are treated before reaching such adverse outcomes, because challenge strain and dose are controlled for, challenge studies provide an excellent opportunity to identify host factors relating to disease severity. Therefore surrogate measures of severe disease must be used. In the Oxford human challenge studies, volunteers with an oral temperature above 40°C or a grade 4 laboratory abnormality are considered to have "severe" enteric fever (Gibani, Jones, et al. 2019). However as this dichotomous outcome does not take into account the whole spectrum of acute disease, previous analyses on both the Maryland and Oxford human challenge studies have focused on continuous measures of severity. In a re-analysis of the Maryland human challenge studies, peak temperature, duration of temperature above 39°C and symptom score were used as measures of severity, and were weakly correlated with dose (Glynn et al. 1995). In the Oxford human challenge models, symptom score and bacterial load have been used to assess the effect of serum bactericidal antibody on attenuating severe disease (Juel et al. 2018), and maximum CRP concentration, maximal temperature change from baseline and duration of bacteraemia in order to relate transcriptional perturbation with clinical phenotype (Blohmke et al. 2016).

Correlations between all cytokines and markers of disease severity are summarised in Figure S3.7. Log fold changes in the MFIs of IL1RA and IP10 relative to baseline were correlated with both temperature ($\rho = 0.56$, $p = 0.0005$ and $\rho = 0.58$, $p = 0.0002$, respectively at time of diagnosis, including non-diagnosed participants at Day 10; Pearson correlation) and summed self-recorded severity scores for all non-fever related symptoms ($\rho = 0.64$ and $\rho = 0.65$, $p > 0.05$ as few symptom scores were recorded at this time point) (Figure 3.20a). Furthermore, before adjusting for multiple testing IP10, IL1RA, MIG were significantly higher at time of diagnosis in those who were defined as having severe disease relative to those who had mild/moderate disease (Figure S3.8; $p < 0.05$, linear modelling). However, IP10, IL1RA and MIG

levels at time of diagnosis displayed a trend towards a positive correlation with duration of fever and bacteraemia, suggesting upregulation of these molecules did not have a protective effect, while IL23 displayed a trend towards a negative correlation (ρ = -0.19, p = 0.3 with duration of blood culture positivity).

Figure 3.20: a. Correlation between the $\log_2$(Fold change in MFI) of IL1RA, IP10 and MIG and temperature, symptom score and C-reactive protein. Pearson correlation coefficients (ρ) are shown on a blue-red continuous scale. White asterisks indicate whether ρ is significantly different from 0 (p < 0.001 ***, p < 0.01 **, p < 0.05 *). NAs indicate an insufficient number of measurements.
b. Correlation between IFN-induced ligands IP10 and MIG, and the cytokines thought to induce them, IFNB, IFNG and TNFA, over all participants. Pearson correlation coefficients (ρ) are shown on a blue-red continuous scale. White asterisks indicate whether ρ is significantly different from 0 (p < 0.001 ***, p < 0.01 **, p < 0.05 *).

a. b.

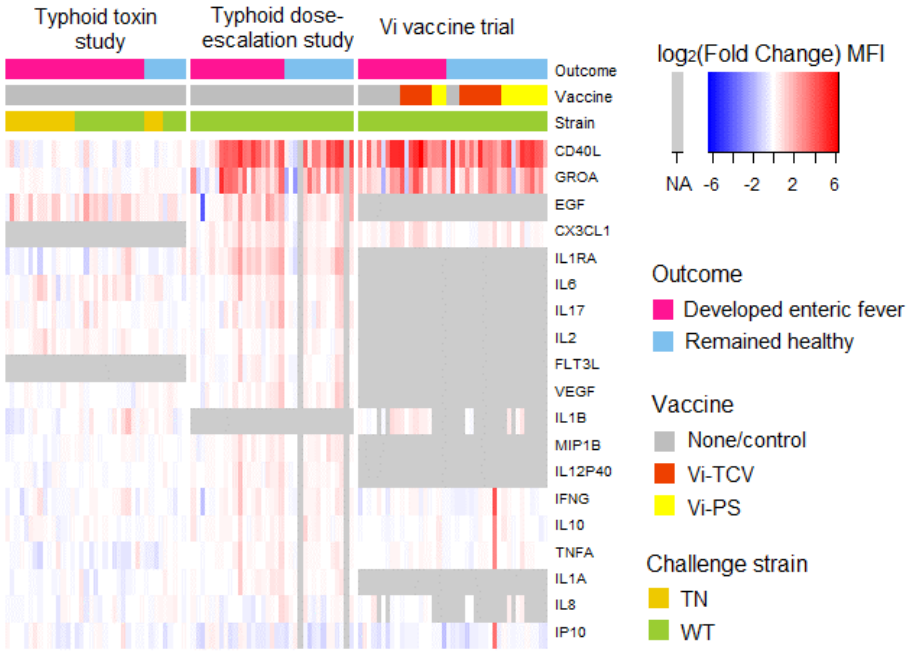


As MIG and IP10 are both IFN-inducible CXCR3 ligands, correlation between these cytokines and the cytokines thought to induce their expression (IFN-γ, IFN-β and TNFA) was assessed (Figure 20b.). IP10 levels were most closely correlated with IFN-γ (ρ = 0.3, p = 1.3 x 10^{-8}) and MIG with TNFA (ρ = 0.3, p = 1.3 x 10^{-9}). There was a significant overall correlation between IL1B and IL1RA (p = 0.01 without adjustment, Figure S3.9) with a ρ of 0.45 at time of diagnosis. IL1RA was correlated with other cytokines at each time point to assess potential candidates for IL1RA-

mediated downregulation. IL1RA was overall negatively correlated with MCSF, GROA, SDF1A, IL2, VEGFD, RANTES, IL22, PAI1, IL21, BDNF AND IL23 (FDR < 0.05, Figure S3.10).

As with the 12 hour time point, when the average $\log_2$(Fold Change MFI) in acute disease relative to baseline was compared with that from the typhoid dose finding study and Vi vaccine trial, there was a negative bias which increases linearly with $\log_2$(Fold Change MFI) (Figure 3.21). While the rise in IP10 during acute disease was consistent, the eBioscience Luminex kit did not detect an expected rise in IFN-γ. Across all time-points, IP10 and IL1RA were also significantly associated with body temperature and C-reactive protein in the typhoid dose-escalation study and Vi vaccine trial ($\rho > 0.1$, $p < 1 \times 10^{-5}$). However, this trend is less clear when limiting samples to those within the first two days after typhoid diagnosis (Figure 3.22, Figure S3.11). The rise in IP10 during acute disease, the only severity biomarker also measured in the Vi vaccine trial, interestingly appeared to be mildly attenuated by vaccination ($p = 0.04$ for the control versus the Vi-TCV group, at 96 hours post-diagnosis using a Mann-Whitney-Wilcoxon test; Figure 3.23).

Figure 3.21: a. Heat map summarising the log2(Fold Change MFI)s of each participant during acute disease (12-24 hours after diagnosis in the typhoid toxin study, 12 hours after diagnosis in the Vi vaccine trial, and 48 hours after diagnosis in the dose escalation study).
b. Bland-Altman plots showing the difference in the log_2(Fold change MFI) for each cytokine between studies, and how this relates to the mean log_2(Fold change MFI) at 12 hours post challenge and after diagnosis (12-24 hours after diagnosis in the typhoid toxin study, 12 hours after diagnosis in the Vi vaccine trial, and 48 hours after diagnosis in the dose escalation study). A regression line with 95% confidence intervals is shown in grey. The bias is shown in yellow, with 95% confidence intervals illustrated by a bar. A confidence interval crossing y=0 indicates no significant bias. The upper and lower limits of agreement and corresponding 95% confidence interval are shown in blue.
a.



b.

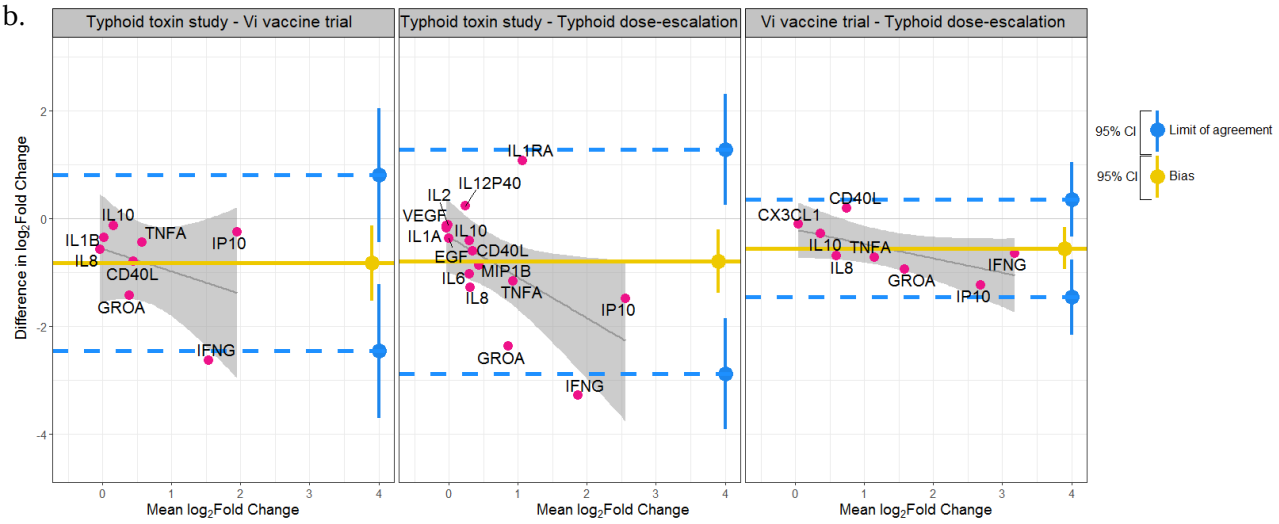


Figure 3.22: Correlation between the log(Fold Change MFI) of IP10 and IL1RA with temperature and C-Reactive Protein amongst participants who developed typhoid for each study. Scatterplots are shown both including all time points and with samples limited to those collected within the first 2 days after typhoid diagnosis. Pearson's product-moment correlation coefficient (ρ) and the p-value for a test of whether ρ is different from zero are shown above each scatterplot. Points are coloured by how many days from the time of diagnosis the sample was collected.

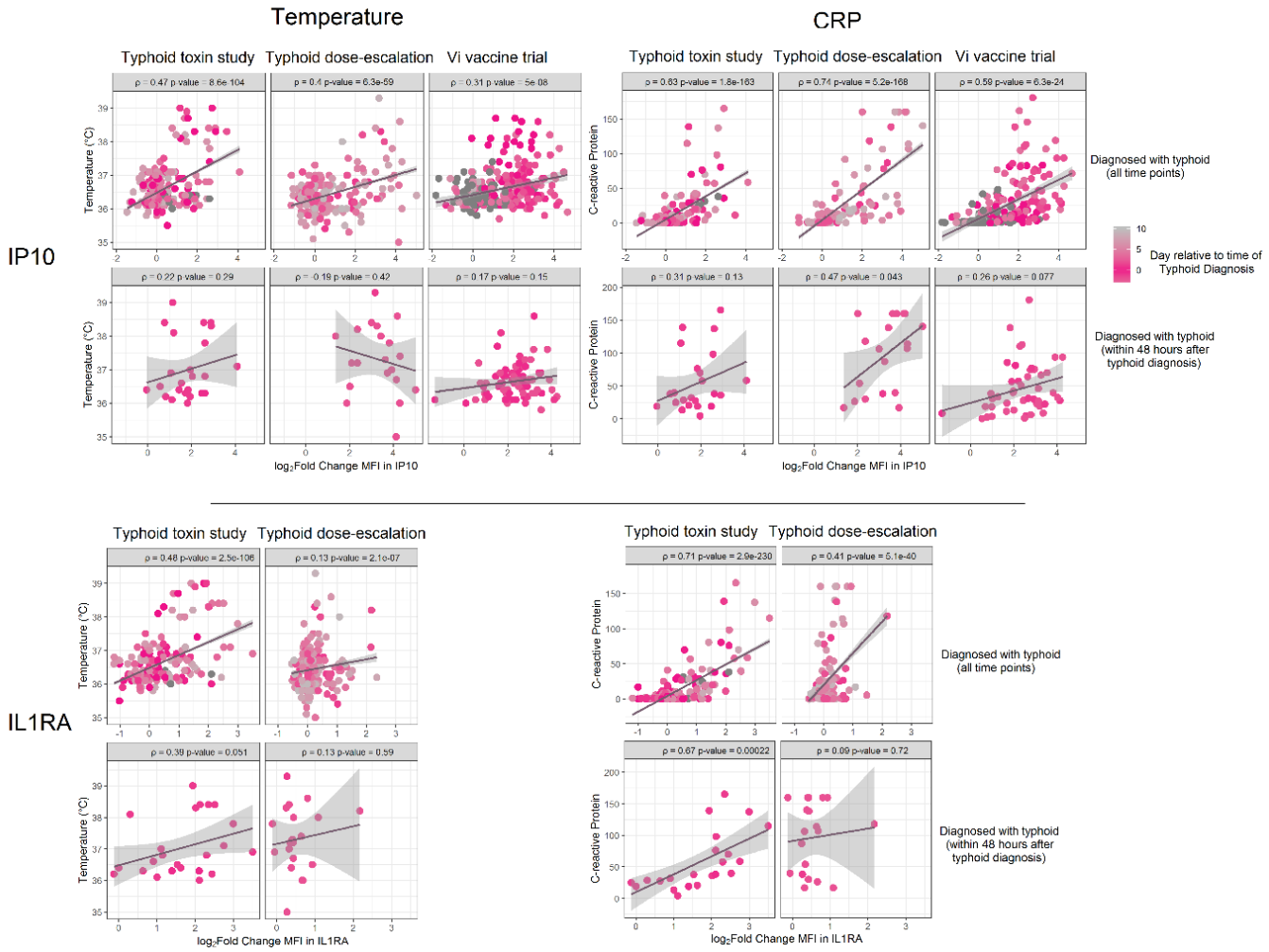
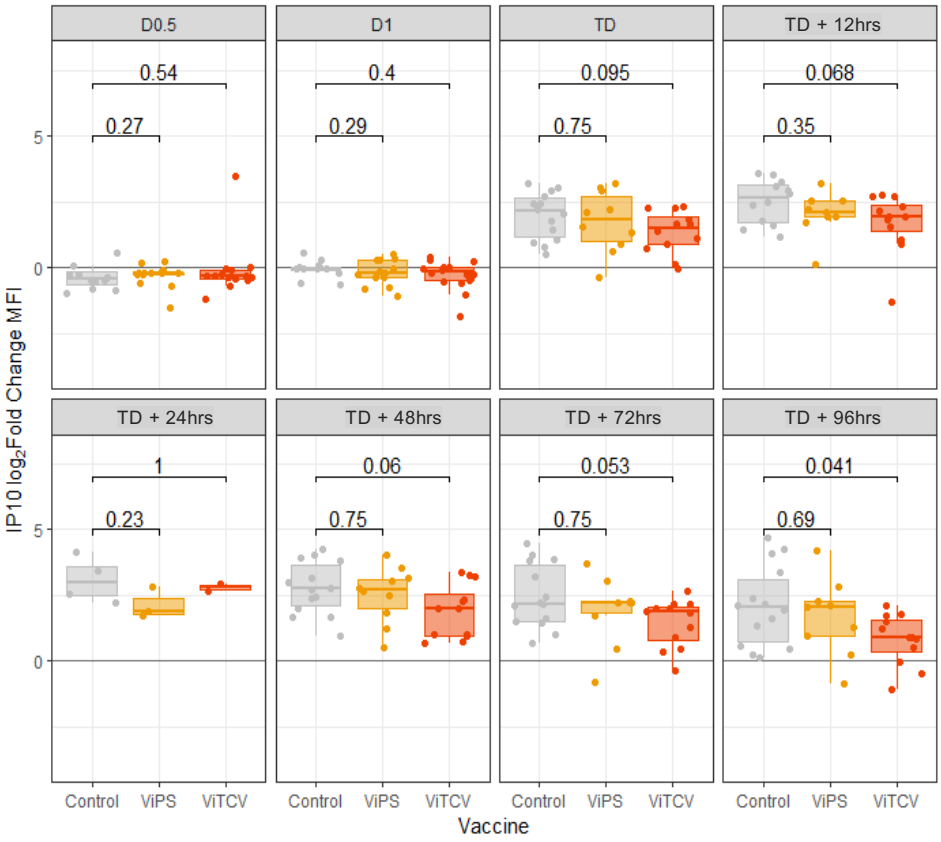


Figure 3.23: Boxplots comparing the $\log_2$(Fold Change MFI) in IP10 at 12 hours post-challenge between vaccine groups. Significance was calculated using a Mann–Whitney–Wilcoxon test.



3.4 Discussion

3.4.1 Changes in plasma cytokines 12 hours post-challenge

12 hours post-challenge 11/62 cytokines were significantly changed ($p < 0.05$), consistent with previous evidence of a cytokine signal at this time point (Blohmke et al. 2016; Darton et al. 2017). At this stage, plasma cytokine profiles were similar between those who did and did not go on to develop typhoid fever, suggesting this transient change is not protective, but also not necessarily indicative of bacterial invasion. The marked rise in EGF was consistent with previous studies and in this new dataset TGFA, another EGFR ligand, also rose. EGFR is expressed by macrophages and is thought to regulate their activation and pro-inflammatory cytokine production in response to gastrointestinal bacterial pathogens (Hardbower et al. 2016; MacDonald and Zaiss 2017). In humans, EGFR inhibitors increase susceptibility to bacterial infections, suggesting this pathway plays a non-redundant role in anti-bacterial immunity (Burtness et al. 2012; Wang et al. 2017).

The second most upregulated cytokine was brain-derived neurotrophic factor (BDNF), which promotes the survival of neurons (Cattaneo et al. 2016). This increase was validated by ELISA, and is not observed in unchallenged controls. BDNF is expressed not only by the brain but also by leukocytes (Cattaneo et al. 2016), and is released by PBMCs in response to LPS (Lommatzsch et al. 2007) and human monocytes in response to IL6 and TNF-α (Schulte-Herbrüggen et al. 2005). In mice BDNF was shown to maintain the integrity of the colonic epithelium through modulation of tight junctions (Li, Cai, and Yan 2018). BDNF is elevated in the serum and cerebrospinal fluid of patients with bacterial meningitis (Chiaretti et al. 2004; Morichi et al. 2013), and in the plasma of patients with lower respiratory tract infections and H1N1 serotype influenza (Chiaretti et al. 2013). However, a BDNF response to a gastrointestinal infection has not previously been observed. Two cytokines belonging to the common γ chain family, IL2 and IL15, were also to a lesser extent upregulated, and play a role in the induction of T cell responses (Akdis et al. 2016). Interestingly, the gene encoding IL2 receptor subunit *IL2RG* is specifically

upregulated in HEK 293T cells in response to *S.* Typhi but not *S.* Typhimurium stimulation (Hannemann and Galán 2017). While the rise in IL2 was not replicated in an independent multiplex cytokine assay, in future a targeted ELISA could address this discrepancy.

3.4.2 Potential sources of the early cytokine signal

Cell stimulation *in vitro* was used to identify potential sources of the cytokines observed in the early response to challenge. Consistent with post-challenge data from the eBioscience Luminex assays, white blood cells produced EGF, IL6, IL2 and TGF-α in response to *S.* Typhi, and consistent with the post-challenge data from the Millipore Luminex assays there was a rise in GRO-α, EGF and IL1RA. BDNF, while not reaching significance, was consistently raised in the supernatants of stimulated leukocytes relative to unstimulated controls for each of three donors. Soluble CD40L, which is raised 12 hours post-challenge when measured using the Millipore Luminex assays, appeared to fall at 8 hours relative to baseline even in unstimulated cells. Furthermore, in contrast to the eBioscience Luminex data suggesting a small fall in TNF 12 hours post-challenge, and the Millipore Luminex data suggesting no change, in stimulated white blood cells there is a marked rise in response to infection. Likewise IL8 is produced in response to *in vitro* infection but not early *in vivo* infection. Thus while white blood cells are capable of producing EGF, BDNF, IL6, IL2, TGF-α, GRO-α and IL1RA in response to *S.* Typhi infection, and are therefore a feasible source of these cytokines *in vivo*, *in vitro* stimulation generates a more inflammatory cytokine profile than detected in plasma following human challenge. Given the lack of correlation previously observed between cytokines induced by *in vivo* endotoxemia and *in vitro* stimulation of whole blood with LPS (Dorresteijn et al. 2010), this discrepancy is not unexpected, and could be due to a number of factors. Firstly, an MOI of 10 almost certainly results in greater exposure of white blood cells to *S.* Typhi than in the blood or intestinal mucosa *in vivo*, and certain cytokines may be more dose-dependent than others (Patrone, Bish, and Stein 2014). Secondly, cytokines in the plasma may be raised due to "spill over" of cytokines secreted by tissue-resident cells or cells migrating from the intestinal

mucosa into the blood, populations which may have different properties to peripheral blood cells directly interacting with bacteria. Thirdly, cytokine stability may be altered in an *in vivo* environment such as tissue fluid or plasma compared with in culture media (Zhou et al. 2010). Finally, it may be that under the conditions of the *in vitro* cell stimulation *S.* Typhi is expressing fewer anti-inflammatory virulence factors such as the Vi capsule than *in vivo* (R. P. Wilson et al. 2008).

Despite disparities between *in vitro* and *in vivo* responses to early infection, further *in vitro* experiments were carried out to investigate which specific cell types may be responsible for secretion of "early response" cytokines. Consistent with *in vitro* stimulation experiments carried out on a model of the intestinal mucosa including epithelial and immune cells (Salerno-goncalves et al. 2019), IL6 and TNFA were primarily produced by $CD14^+$ cells in response to *S.* Typhi. CD40L, EGF, IL2 and IL1RA on the other hand were only significantly produced by granulocytes. Neutrophil chemoattractants GRO-α and IL8 were produced by granulocytes, monocytes and lymphocytes. While previous studies have identified epithelial cells as a major source of IL8 following exposure to *S.* Typhi (Salerno-goncalves et al. 2019), no marked IL8 response was observed in infected intestinal organoids. Secretion of other cytokines was also limited, with organoids derived from just one of the three donors exhibiting an increase in GRO-α and TGFA. Given this model does not contain immune cells and *S.* Typhi does not cause gastroenteritis, the absence of changes in cytokine profile is not unexpected. It is also highly likely that the MOI is not comparable to either the stimulated white blood cells or *in vivo* infection, as organoids are three-dimensional structures consisting of many cells which are difficult to count precisely. Of the cytokines assayed here, four have previously been characterised in the supernatants of intestinal biopsies stimulated with *S.* Typhi. While there was consistently no secretion of IL6, IL8 or TNFA, intestinal biopsies do secrete IL4 from the apical side following infection (Nickerson et al. 2018). In addition to the peripheral white blood cells and intestinal cells investigated here, there are further organs *S.* Typhi may interact with

in vivo which could contribute to early changes in cytokine profile, such as the liver (Albayrak, Gunbey, and Aktas 2011; El-Newihi, Alamy, and Reynolds 1996), spleen (Dunlap et al. 1991) and lymph nodes.

3.4.3 The effect of serovar and Vi expression on secretion of early response cytokines

To investigate the relevance of microbial factors such as the Vi capsule to cytokine secretion, leukocytes were stimulated with a variety of *Salmonella* strains *in vitro*. Of the cytokines profiled, IL6, IL8 and TNFA have previously been measured following stimulation of an organotypic intestinal model with wild-type *S.* Typhi, *S.* Typhi vaccine candidate strains and *S.* Paratyphi A (Salerno-Gonçalves et al. 2018; Salerno-goncalves et al. 2019). We replicate the findings from this model that *S.* Typhi strain CVD 909 induces (non-significantly) higher levels of IL8 and lower levels of IL6 than parent strain CVD 908. Compared with CVD 908, CVD 909 constitutively expresses the Vi capsule, but lacks heat shock protein *htrA*, limiting its replication in host tissues. While the lower levels of IL6 induced by CVD 909 can be explained by expression of the Vi capsule (R. P. Wilson et al. 2008), the higher level of IL8 secretion is inconsistent with previous evidence that the Vi capsule suppresses IL8 secretion while the *htra* mutation does not affect it (Lowe et al. 1999; Raffatellu et al. 2005; R. P. Wilson et al. 2008). Expression of the Vi capsule by *S.* Typhimurium significantly decreased IL6 secretion relative to stimulation with wild-type *S.* Typhimurium, with a trend towards suppression of IL8 secretion ($p = 0.2$ at 4 hours post-inoculation), while culture of *S.* Typhi Quailes in low osmolarity conditions in order to induce Vi expression did not affect cytokine responses. Also in agreement with the organotypic intestinal model, we find that *S.* Paratyphi A induces higher levels of IL6 and TNFA than *S.* Typhi, possibly as a result of lacking the Vi capsule. Amongst the cytokines not previously characterised in *in vitro* typhoidal *Salmonella* infections, a greater amount of anti-inflammatory IL1RA was produced in response to *S.* Paratyphi A and *S.* Typhimurium than *S.* Typhi Quailes strain.

3.4.4 Correlates of fever and symptomology in acute disease

IP10, MIG and IL1RA were upregulated in acute disease, and more so in those defined as having severe typhoid fever. They were also positively correlated with oral temperature, symptom score and C-reactive protein, but not with quicker resolution of disease. IP10 and MIG are both IFN-γ induced CXCR3 ligands. In previous typhoid challenge studies, CXCR3 expression on T_{reg} cells increased in response to challenge (McArthur et al. 2015). Despite previous literature suggesting MIG is induced by IFN-γ alone while IP10 is induced by IFN-γ, IFN-α/β and TNF (Groom and Luster 2011), here MIG correlated better with TNF and IFN-β than IFN-γ. The main role of IP10 and MIG is the chemo-attraction of T cells, macrophages, dendritic cells, neutrophils and NK cells towards sites of inflammation, as well as inducing proliferation or apoptosis (Ichikawa et al. 2013; Mingli et al. 2011). MIG also has direct antibacterial properties (Reid-Yu et al. 2015). IP10 and MIG have been associated with disease severity in acute respiratory infections (Hayney et al. 2017), melioidosis (Lauw et al. 2000), tuberculosis (Hasan et al. 2009), hepatitis C infection (Esmon, Xu, and Lupu 2011), and dengue fever (Ferreira et al. 2015). However, it is currently unclear whether IP10 and MIG play a protective role in bacterial infection, or directly contribute to disease severity. On the one hand, CXCR3 knock-out in mice results in decreased neutrophil recruitment to the gut following oral *S.* Typhimurium challenge, resulting in greater bacterial loads and inflammation in the spleen and liver (Chami et al. 2017). On the other hand, treatment of mice with anti-CXCR3 immunoglobulin has been found to improve sepsis survival (Herzig et al. 2012), while knock-out of CXCR3 improves survival of bacteraemic pneumonia (Seyoum, Yano, and Pirofski 2011). Furthermore, in a challenge study where human volunteers were given a live attenuated influenza vaccine followed by *Streptococcus pneumoniae* inoculation, high IP10 levels at time of inoculation were associated with impaired innate control of bacterial density (Jochems et al. 2018).

IL1RA acts as an anti-inflammatory cytokine by competing with the inflammatory cytokines IL1α/β for the IL1 receptor, binding without activating downstream inflammatory pathways

(Arend 2002). IL1RA is secreted both as an acute phase protein by the liver, and by monocytes, macrophages and neutrophils. In humans, IL1RA has previously been found to significantly correlate with bacterial load in tuberculosis (Heslop et al. 2016). It may be that in individuals with more severe disease, there is a greater increase in IL1RA to prevent excessive inflammation and limit the duration of fever (Cartmell et al. 2001). Another possibility is that IL1RA is impairing IL1α/β-dependent bacterial clearance, ultimately increasing severity. In mouse models, treatment with IL1RA increases both mortality from *Staphylococcus aureus* sepsis (Ali et al. 2015) and bacterial load in pneumococcal pneumonia (Rijneveld et al. 2003). Furthermore, IL1RA knock-out mice are less susceptible to *Listeria* infection (Hirsch et al. 1996).

One difficulty in human challenge studies is assessing the extent to which factors such as vaccination or previous exposure attenuate disease severity. Symptom scores are subjective and variable between volunteers, while temperature measurements only capture one feature of clinical disease. IP10, MIG and IL1RA may be good candidates as biomarkers for disease severity, and may improve comparability between volunteers and studies. The results presented also raise the question as to whether targeting these pathways, for example with anti-CXCR3 immunoglobulin, may be a promising avenue for improving the outcome of severe typhoid fever.

3.4.5 Differences between challenge strain groups

Although there were no significant clinical differences between challenge strain groups in the typhoid toxin study, of those who were diagnosed with typhoid fever 1/15 in the wild-type group and 4/15 of those in the toxin-negative developed “severe” typhoid fever, defined as oral temperature ≥40°C or a grade 4 laboratory abnormality. We therefore hypothesised that cytokines correlating with fever and symptom score may be elevated in the toxin-negative group relative to the wild-type group.

However, there was no significant difference in IP10, MIG, or IL1RA between the two groups. Furthermore, over the course of the human challenge period, no principal component or unsupervised hierarchal clustering was observed by challenge strain group, suggesting that this study did not capture major differences in cytokine profile between those challenged with toxin-negative and wild-type *S.* Typhi. This result may reflect the lack of significant differences between the clinical profiles of the two groups, as well as the lack of differences in cytokine profile between leukocytes stimulated *in vitro* with the two strains. The cytokine with the greatest overall difference between groups appeared to be RESISTIN. Although RESISTIN is best known for its role in insulin resistance (Jamaluddin et al. 2012), it also acts as a pro-inflammatory cytokine, released by neutrophils in response to LPS and bacterial stimulation (Johansson et al. 2007; Snall et al. 2016).

3.4.7. Discrepancies between multiplex cytokine assay platforms

Overall, in those who developed disease cytokines were most perturbed from baseline 12-24 hours after typhoid diagnosis, followed by time of diagnosis, Day 14 and 12 hours post-challenge. There was relatively low agreement between results of the assay used for the typhoid toxin study and those used for previous typhoid challenge studies, with only 5/9 of the cytokines tested on both kits showing significant correlation. Poor concordance between results from assays produced by different Luminex manufacturers has been observed previously (Berthoud et al. 2011). In the typhoid toxin study the sensitivity to certain high abundance cytokines was low compared with assays from other manufacturers. Possible explanations for this difference include the use of different antibodies for cytokine detection or the need to make compromises in order to optimise the assay for a larger cytokine panel.

3.4.7. Conclusion

To summarise, plasma cytokine profiles are perturbed during acute disease and 12 hours post-challenge, but not during the intervening incubation period, supporting the idea that *S.* Typhi

"hides" from the immune system within the spleen, liver or bone marrow during this time. No major differences were observed between challenge strain groups, reflecting the similar clinical outcomes and suggesting that the typhoid toxin does not play a major role in the inflammation induced by acute disease. We found discrepancies between the results given by multiplex cytokine assay kits produced by different manufacturers, and therefore recommend validation by ELISA or a different kit. BDNF and EGF were raised 12 hours post-challenge in two different assays, and may represent interesting targets of further study into early host pathogen interactions. As both were produced by white blood cells stimulated with *S.* Typhi, this *in vitro* model would be suitable for further mechanistic experiments, and as demonstrated here may be used to investigate the effect of bacterial virulence factors on host responses. IP10, MIG and IL1RA were identified as correlates of fever and inflammation, and may be valuable as objective biomarkers for the attenuation of disease severity by vaccination or drug treatment. It is currently unclear whether early response cytokines and severity biomarkers are protective, contribute to pathology, or are by-products of infection. Knock-out of the genes encoding these cytokines, or addition of recombinant cytokines, will be required to determine their direct effects.

Chapter 4 | Changes in the blood transcriptome within the first 24 hours of typhoid challenge

4.1 Introduction

Transcriptomics has previously been used an unbiased approach to characterise pre-symptomatic host responses to challenge with influenza (Huang et al. 2011), rhinovirus (Proud et al. 2008) and malaria (Ockenhouse et al. 2006), providing a broader snapshot of the pathways activated post-infection than plasma cytokines alone. While transcriptomics has been applied to dissect host-pathogen interactions during acute enteric fever and in *in vitro* models (see table 4.1), host transcriptional responses during the incubation period of enteric fever, which lasts 1-3 weeks in the field, and eight days in the Oxford human challenge model (Awofisayo-Okuyelu et al. 2018; Olsen et al. 2003), have not yet been explored.

In the field it is not possible to study infected patients before clinical presentation, and therefore little is known about host-pathogen interactions during this time period. In a human challenge model of typhoid, participants who do not develop typhoid on their first exposure are less likely to do so on their second exposure compared with participants who develop disease (unpublished, Figure 4.1), suggesting certain individuals may have an innate resistance to infection. Furthermore, in the first three days after challenge *S.* Typhi is detected in the stool of both participants who go on to develop clinical disease and in participants who stay healthy (Darton et al. 2016). Likewise, *S.* Typhi DNA has been detected in the blood of both those who later develop typhoid and those who do not in the first few days after challenge (Darton et al. 2017), as has a transient cytokine signal (Blohmke et al. 2016; Chapter 3 of this thesis). This suggests that despite evidence of bacterial exposure and survival in all who are challenged, some participants are able to raise a protective immune response and evade typhoid fever. We applied transcriptomics to investigate the nature of the early host response to *S.* Typhi challenge using an unbiased approach.

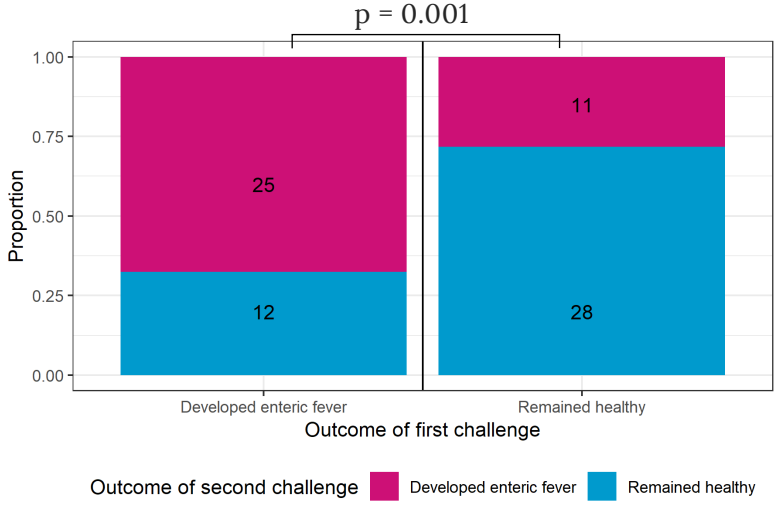
Here we identify 216 non-circadian genes differentially expressed 12 hours post-challenge. Gene sets associated with innate immunity are identified as being over-represented, with an estimated rise in the proportion of neutrophils which returns to baseline after 24 hours. Responses were similar regardless of outcome of infection, challenge strain or vaccination status.

Table 4.1: Published literature assessing changes in the transcriptome in enteric fever and *in vitro* typhoidal *Salmonella* stimulation

Human studies				
Cohort	Method	Timepoints	Findings	Reference
Vietnamese hospital patients with culture-positive typhoid fever (n = 29)	Stanford Human 42K cDNA array	Day of hospital admission, Day 3, 9 and 28 after admission, 9 months post-admission	Transcriptional profiles of acute typhoid, convalescent typhoid and controls are distinct from one another. Two upregulated clusters of genes in acute disease were correlated with temperature and % neutrophils, while another contained genes induced by IFN-γ.	(Thompson et al. 2009)
Patients in Bangladesh with fever and blood culture confirmed paratyphoid (n = 3)	Selective Capture of Transcribed Sequences and cDNA microarray to detect bacterial RNA	Acute disease	*S.* Paratyphi A transcripts can be detected in the blood of patients, and have a distinct transcriptional profile to *S.* Paratyphi A in culture.	(Sheikh et al. 2010)
Patients in Bangladesh with fever and blood culture confirmed typhoid (n = 5)	Selective Capture of Transcribed Sequences and cDNA microarray to detect bacterial RNA	Acute disease	*S.* Typhi transcripts can be detected in the blood of patients, and have a distinct transcriptional profile to *S.* Typhi in culture.	(Sheikh et al. 2011)
Sub-study of the Community-Acquired Bacteraemic Syndrome in Young Nigerian Children surveillance program (n=6)	Agilent 4 × 44K array	Acute disease (before antibiotic treatment) and convalescence (4 weeks after presentation)	Transcriptional profiles of acute and convalescent typhoid are distinct. *S.* Typhi infection induces a distinct signature to other bacteraemic infections, including genes involved in ligand binding, and lipocalin-2.	(Khoo et al. 2011)
Typhoid dose finding human challenge study (n = 41)	Illumina HT-12v4 bead-array	Baseline, 24 hours before diagnosis, at time of diagnosis, 24	IFN, neutrophil and apoptosis related gene modules were over-expressed during acute disease. Inflammatory	(Blohmke et al. 2016)

		hours after diagnosis, Day 14 post-challenge	modules were associated with temperature and CRP.	
Paratyphoid dose-finding study (n = 1) and re-challenge study (n = 1)	TCR sequencing (Illumina MiSeq)	Acute disease	There is evidence of clonotype expansion in T cells targeting CdtB, PhoN and HlyE. The TCRβ repertoire in $CCR7^{-}CD38^{+}$ cells was less diverse than $CD38^{-}$ $CCR7^{-}$cells	(Napolitani et al. 2018)
Typhoid dose finding study and Typhoid oral vaccine trial (n = 61)	Illumina HT-12v4 bead-array	Baseline, 12 hours and 24 hours post-challenge	The following thesis chapter	
Paratyphoid dose finding study and Vi vaccine trial (n = 103)	Illumina HiSeq 200 V4	Baseline and 12 hours post-challenge		
In vitro models of typhoidal *Salmonella* infection				
Cell types	Method	Conditions	Findings	Reference
THP-1 and Hep2 cells	Illumina Human HT-12_V4 Beadchips	Infected with wild-type, Hj^{+}, $Hz66^{+}$, $fliC^{-}$ and $invA^{-}$ *S.* Typhi strain BRD948 at MOI 10 for one hour	In both cell lines, Hd (the wild-type flagellin) and Hj flagellin gave similar, more inflammatory responses, while Hz66 induced fewer differentially expressed genes and gave a response more similar to the fliC and invA knockouts.	(Schreiber et al. 2015)
HEK 293T and and Henle-407	Illumina HiSeq 2500 system	Infected with ISP2825 strain *S.* Typhi at MOI 100 or SL1344 strain *S.* Typhimurium at MOI 20 for 4 hours	*S.* Typhi infection induces higher levels of *IL8* and *EGR1* and more activation of NF-κB signalling pathways than *S.* Typhimurium.	(Hannemann and Galán 2017)
Intestinal biopsies	Illumina HiSeq 2500 system	Infected with 1×10^{8} CFU Ty2 strain *S.* Typhi for up to 2 hours	*GSTM1, CRIP1* and chemokine *CCL25* were upregulated, while genes relating to B-cell receptor signalling were downregulated	(Nickerson et al. 2018)

Figure 4.1: Proportion of those in the enteric fever re-challenge study (see Chapter 2.2.5) diagnosed with enteric fever following second challenge with *S.* Typhi or *S.* Paratyphi A, stratified by outcome of first challenge. The p value for a Pearson's Chi-squared Test is shown.



4.1.1 Research questions

In the following chapter we attempt to address the following research questions with regards to the transcriptional response in the first 24 hours of enteric fever challenge:

1. Question: Given there are early changes in plasma cytokines and evidence of an asymptomatic bacteraemia, can we detect statistically significant transcriptional changes in the blood 12 hours post-challenge?

   Approach: Transcriptional profiling by microarray is carried out on two challenge cohorts, allowing us to screen a large number of genes for changes in expression. We use publicly available data from a circadian rhythms study as a control cohort to account for changes attributable to circadian rhythms. In order to identify reproducible changes, results are validated using two independent challenge cohorts transcriptionally profiled by a different method (RNA-sequencing).

2. Question: Is any transcriptional perturbation observed evidence of an early innate response to infection?

   Approach: Gene set enrichment and pathway analyses are carried out to functionally annotate differentially expressed genes, allowing us to investigate whether transcriptional changes likely reflect an early immune response.

3. Question: Are any changes in gene expression due to changes in white blood cell populations, or transcriptional regulation?

   Approach: Changes in gene expression are correlated with that of cells stimulated with *S.* Typhi *in vitro* to identify genes transcriptionally upregulated. Deconvolution is used to identify any predicted changes in cell subsets early after challenge. In order to assess whether these different cell subsets are able to take up, respond to and kill *S.* Typhi, we use flow cytometry and intracellular survival assays. We then investigate whether

virulence factors such as the Vi capsule and typhoid toxin affect interactions between leukocytes and typhoidal *Salmonella* serovars.

4. Question: Is it possible to predict who will develop enteric fever from the early transcriptional response?

   Approach: Differential gene expression is carried out to investigate whether the early response to challenge is significantly different between the two outcome groups. Principal component analysis is carried out based on the most differentially expressed genes between groups to assess whether they are capable of distinguishing between outcome groups. Finally, we compare the magnitude of the transcriptional responses in the groups in order to investigate whether the extent of transcriptional perturbation relates to outcome of challenge.

5. Question: Are there any differences in early transcriptional responses between *S.* Typhi or *S.* Paratyphi A challenge, or vaccination groups?

   Approach: Differential gene expression and gene set enrichment analysis is carried out to identify functionally related sets of genes regulated differently between groups 12 hours post-challenge relative to baseline.

6. Question: Do these transcriptional changes in the blood reflect inflammation in the gut, or direct interactions between peripheral blood cells and bacteria in the blood during primary bacteraemia?

   Approach: Differential expression 12 hours post-challenge is correlated with transcriptional changes in the blood in inflammatory bowel disease, giving us an idea of how blood gene expression changes in response to intestinal inflammation, and transcriptional changes in THP-1 cells directly stimulated with *S.* Typhi *in vitro.*

7. Question: Are the “early response” cytokines from Chapter 3 upregulated at the transcript level in the blood 12 hours post-challenge, and if not, in which tissue are they likely to be expressed?

   Approach: Changes in the gene expression of early response cytokines at the transcript level are correlated with changes in plasma protein levels in order to assess the agreement between the two. Publicly available data on changes in gene expression in different tissues following *S.* Typhi or *S.* Typhimurium infection, or in inflammatory bowel disease, is used to hypothesise where cytokines not upregulated in the blood may originate from.

4.2 Materials and methods

4.2.1 Study design and participants

The primary datasets analysed here were derived from four human challenge studies, outlined in Table 4.2 and discussed in more detail in 2.2.1-4. In the oral vaccine trial only samples from the participants in the control arm were used in this analysis.

4.2.2 Sample processing and genome alignment

3ml peripheral blood was collected in Tempus Blood RNA tubes by clinical staff before challenge (~6-9am) and approximately 12 hours and 24 hours following challenge. Total RNA was extracted using the Tempus Spin RNA Isolation kit (Life Technologies) by Oxford Vaccine Group research staff. The distribution of RNA concentrations, as measured by nanodrop, and RNA Integrity Number for each cohort is shown in Figure S4.1.

Table 4.2: Outline of challenge studies from which transcriptomic data were derived

	Discovery cohort studies					**Validation cohort studies**				
Study	Typhoid dose finding		Typhoid oral vaccine trial			Paratyphoid dose finding		Typhoid Vi Vaccine trial		
Groups	Low dose (n=20)	High dose (n=20)	Placebo (n=30)	3 doses Ty21a (n=30)	One dose M01ZH09 (n=31)	Low dose (n=20)	High dose (n=20)	Control (n=31)	Vi poly-saccharide vaccine (n=35)	Vi tetanus toxoid conjugate vaccine (n=37)
Challenge agent	*S.* Typhi Quailes strain in 30ml sodium bicarbonate solution					*S.* Paratyphi A strain NVGH308 in 30ml sodium bicarbonate solution		*S.* Typhi Quailes strain in 30ml sodium bicarbonate solution		
Dose (CFU)	1-5 × 10^3	1-5 × 10^4	1-5x10^4			0.1-1 × 10^3	1–5 × 10^3	1-5 x 10^4		
Attack rate	11/20	13/20	20/30	13/30	18/31	8/20	12/20	24/31	13/35	13/37
Case definition	Positive blood culture after Day 5, or fever ≥38°C sustained for ≥12 hours		Positive blood culture after Day 7, or positive blood culture after Day 5 with symptoms, or fever ≥38°C sustained for ≥12 hours			Positive blood culture after Day 3, or fever ≥38°C sustained for ≥12 h		Positive blood culture after Day 3, or fever ≥38°C sustained for ≥12 h		
RNA extraction method	Tempus Spin RNA Isolation kit									
RNA profiling method	Illumina HT-12v4 bead-arrays Wellcome Trust Sanger Institute		Illumina HT-12v4 bead-arrays Wellcome Trust Centre for Human Genetics			RNA-seq using Illumina HiSeq 200 V4 Wellcome Trust Sanger Institute				

RNA samples from individuals in the discovery cohort were hybridised onto Illumina HT-12v4 bead-arrays at the Wellcome Trust Sanger Institute, and fluorescent probe intensities captured with GenomeStudio software. Samples were hybridised in three batches (Table S4.1). RNA samples from individuals in the validation cohort were poly-A selected (but not globin-depleted) and sequenced using a HiSeq V4 at the Wellcome Trust Sanger Institute. Fastq files from the same sample were concatenated, and pre-alignment quality control carried out using FASTQC. As all files had consistently high phred scores (>25) across their length, all were

aligned to the human genome (GRCh38 Gencode version 26) using STAR-2.6.1c (Dobin et al. 2013). Total reads per sample ranged from 16-44 million (Figure S4.2). Reads per gene were counted using the STAR GeneCounts mode.

4.2.3 Data processing

The data processing pipeline is summarised in Figure S4.3. A publicly available whole blood transcriptome dataset studying circadian rhythms was downloaded to act as unchallenged controls in the analysis (Gene Expression Omnibus accession number GSE48113) (Archer et al. 2014). As baseline and 24 hour post-challenge samples were taken at 6-9am in the typhoid study, and 12 hour post-challenge samples at 6-9pm, the Archer et al. dataset was subset to in-phase samples at these time points (20 morning samples and 32 evening samples). As samples were transcriptionally profiled using a different platform (an Agilent Whole Human Genome 4 × 44K custom oligonucleotide microarray) much of the upstream processing was carried out separately. Briefly, datasets were background corrected and quantile normalised, then the package lumi used to identify outliers for exclusion. On this basis, two samples in Batch 2 were excluded from the raw data, which were then re-normalised. Probes from each array were aligned to the human genome assembly (GRCh38 GenCode version 26) using STAR-2.6.1c (Dobin et al. 2013). Probes which did not align to genes were excluded, as were probes aligning to multiple genes. Where multiple probes corresponded to one gene symbol, the collapseRows() function from the WGCNA R package was used to select the probe with the highest average mean as being representative, on the basis of this being the most reproducible approach (Miller et al. 2011). Finally, the typhoid challenge and Archer datasets were merged, with any non-overlapping genes excluded. The combined dataset was quantile normalised, batch corrected using the ComBat function from the sva package, and quantile normalised again. Genes corresponding to probes which were not significantly expressed over the negative controls ($p > 0.05$) in more than 37 samples (the number of baseline samples) were excluded.

For the validation cohort, principal component analysis was used for outlier detection, with no samples excluded on this basis. Non-protein coding and haemoglobin subunit genes were excluded. Count tables were filtered to genes with < 1 count per million (cpm) in <31 samples (the number of baseline samples in control participants challenged with *S.* Typhi) and normalised using weighted trimmed mean of M-values scaling (edgeR). Boxplots and principal component analysis plots for each stage of the pipeline are shown in Figure S4.4.

4.2.4 Differential gene expression analysis

For the discovery cohort, a linear regression model was fitted to the microarray data using the limma package, incorporating dose and batch into the model as covariates, and using participant ID as a blocking variable. For the validation cohort, the count matrix was transformed using limma voom, and a linear regression model fitted with vaccination status, sequence pool and dose as covariates and participant ID as a blocking variable.

4.2.5 Pathway analysis and transcription factor binding site over-representation analysis

The Ensembl IDs of significantly (false discovery rate < 0.05) upregulated or downregulated genes were each input into InnateDB. Pathways and transcription factor binding sites over-represented amongst these genes were identified using a hypergeometric test, and p-values adjusted for multiple testing using a Benjamin-Hochberg correction. Pathways from the KEGG, REACTOME, NETPATH, PID NCI, PID BIOCARTA and INOH databases were included, while transcription factor binding sites were all derived from the cisRED database.

4.2.6 Gene set enrichment analysis (GSEA)

Gene lists generated by differential gene expression analysis were ranked by fold change, such that the most upregulated genes were at the top of the list, and the most downregulated at the bottom. The gene lists were then input into a GSEA algorithm (Subramanian et al. 2005), in

order to determine whether members of different blood transcriptional modules (groups of genes related by biological function or cell-specific expression (Li et al. 2013)), were unevenly distributed in the gene list.

4.2.7 Immune cell fraction estimation

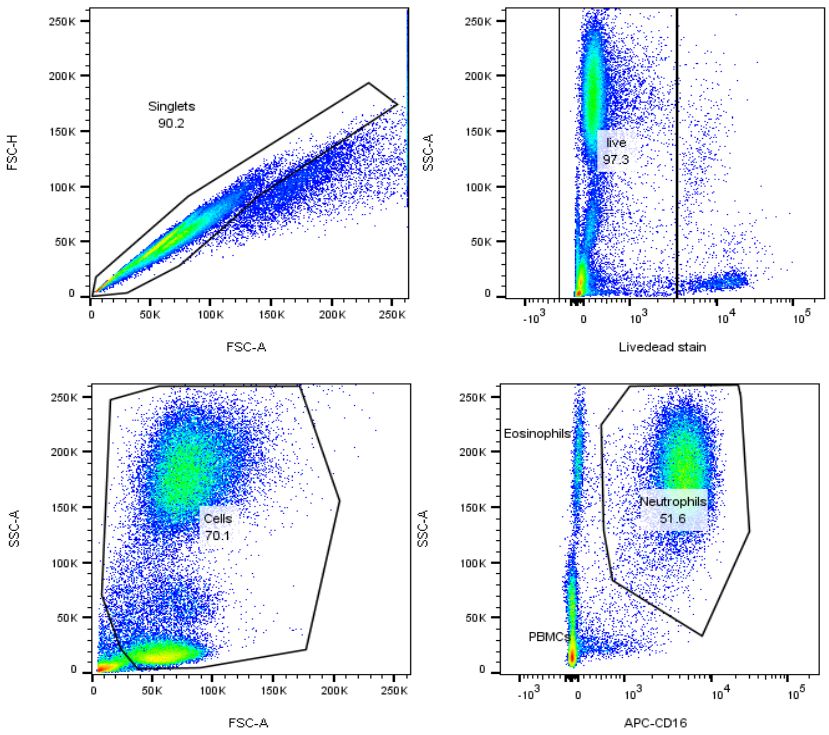
CIBERSORT was used to estimate changes in immune cell populations *in silico*. Estimated cell fractions were compared with differential white blood cell counts taken at baseline in the typhoid dose escalation study.

4.2.8. *In vitro S.* Typhi stimulation and flow cytometry

10ml heparinised blood from three healthy human volunteers was incubated at room temperature for 15 minutes in three volumes of RBC lysis solution (Qiagen). White blood cells were centrifuged at 500G for 5 minutes, and washed with a further three volumes of RBC lysis solution then three volumes of autoMACS Running Buffer. Cells were resuspended in RPMI with 10% FBS at 10^6 cells/ml and seeded at 2 x 10^6 cells/well. A frozen stock of *S.* Typhi (Quailes strain) was thawed, washed twice with RPMI (10% FBS) then diluted to the specified CFU/ml. Cells were stimulated either with *S.* Typhi at an MOI of 10 or 0.1, 100ng/ml *S.* Typhi LPS (L2387, Sigma-Aldrich) as a positive control, or RPMI as a negative control. After one hour extracellular bacteria were killed using 100µg/ml gentamycin. After a further four hours, cells were centrifuged at 500G for 5 minutes. Supernatants were filtered using 0.22µm cellulose acetate Spin-X centrifuge tube filters, while the cells were resuspended in 100µl autoMACS Running Buffer and transferred to a 96 well plate. Cells were then washed with PBS and resuspended in 20µl eBioscience™ Fixable Viability Dye eFluor™ 780 diluted 1:1000 for 15 minutes. Cells were washed with 200µl autoMACS Running Buffer, and then resuspended in an antibody mixture containing BB515 anti-human CD62L (BD Bioscience), PerCP/Cy5.5 CD11b, PE anti-human CD11c (Biolegend), and APC anti-human CD16 (Biolegend) in PBS. Appropriate antibody concentrations were determined by titration. Cells were incubated at 4°C for 20 minutes then

washed twice with autoMACS Running Buffer and once with PBS. Cells were then suspended in 200µl 4% formaldehyde for 30 minutes at room temperature before overnight refrigeration. The following day cells were resuspended in PBS. Fixed cells were filtered by centrifugation in a 96 well 40µm nylon mesh filter plate and transferred to round bottom tubes containing 300µl autoMACS Running Buffer. Flow cytometry was performed using a FACS Aria. Gating was performed in Flowjo V10, with single cells gated based on forward scatter height and area and live cells based on absence of viability dye staining. Viability was 99±2% amongst unstimulated cells, 97±2% amongst cells infected with an MOI of 1, 96±11% amongst cells infected with an MOI of 10, and 97±15% amongst cells stimulated with LPS. Neutrophils were gated based on forward scattering (FSC), side scattering (SSC) and the expression of CD16 (gating strategy is shown in Figure 4.2).

Figure 4.2: Gating strategy for measuring neutrophil activation in response to *S.* Typhi stimulation. Singlets were gated based on forward scatter height and area and live cells based on absence of viability dye staining. Neutrophils were gated based on forward scattering (FSC), side scattering (SSC) and the expression of CD16.



4.2.9 In vitro stimulation with cell supernatants

A 10ml heparinised blood sample was taken from one healthy human volunteer. RBC lysis and washing was carried out as above. Cells were resuspended either in 900µl of the filtered supernatant taken from the previous white blood cell stimulation (see 4.2.8), fresh RPMI with 10% FBS as a negative control, or 100ng/ml *S.* Typhi LPS (L2387, Sigma-Aldrich) in RPMI with 10% FBS as a positive control. Cells were incubated at 37°C for 4 hours, then washed with PBS and transferred to a 96 well plate.

4.2.10 Flow cytometry using clinical samples from challenge participants

Blood samples were taken from participants enrolled in the typhoid toxin study (see Chapter 2.2.6): a double-blinded and randomised outpatient human challenge study designed to investigate the role of typhoid toxin in the pathogenesis of enteric fever. Heparinised blood samples were taken from participants at baseline, 12 hours after challenge and 24 hours after challenge in BD Vacutainer® PST™ Tubes. 1.5ml blood from each sample was transferred to a falcon tube and RBC lysis, washing, staining and fixation carried out as in 4.2.8. This experiment was performed four times, with small variations in protocol outlined in Table S4.2.

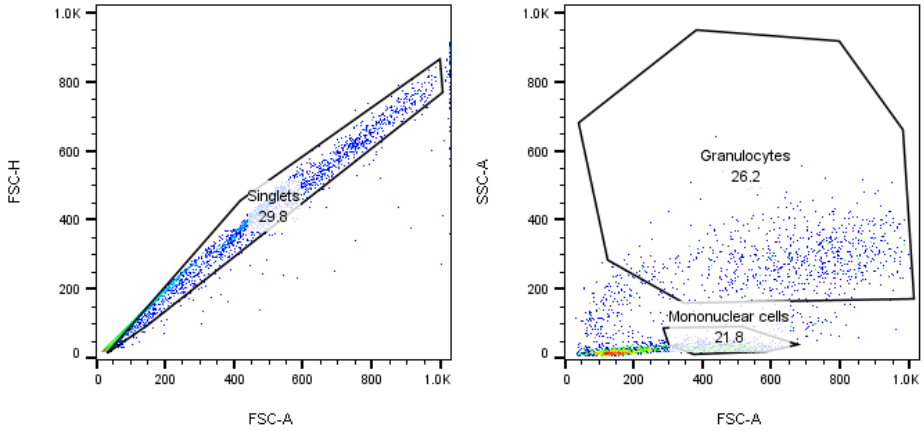
4.2.11 Factors affecting neutrophil activation

Heparinised blood samples from a healthy human donor were collected in either a falcon tube or a Vacutainer® PST™ Tube. To test the effect of temperature, samples were incubated for 2 hours at 4°C, 25°C or 37°C. To test the effect of shaking, samples were either manipulated as little as possible, inverted for 60 seconds, or vortexed for 60 seconds. To test the effect of washing solution, cells were washed using PBS or Hanks' Balanced Salt solution. To test the effect of fluorophore, cells were surface stained using either 1µl FITC anti-human CD62L or 4µl BB515 anti-human CD62L. To test the effect of buffer in the antibody mixture, antibody mixtures containing either PBS or BD Horizon Brilliant™ Stain Buffer were used during staining. The remainder of the protocol was carried out as in 4.2.8.

4.2.12 *S.* Typhi uptake and production of reactive oxygen species

White blood cells from one healthy donor were isolated as in 4.2.8 and seeded in a 96 well plate at a density of 100,000 cells per well in RRMI. 123-Dihydrorhodamine was diluted 1:10 in PBS and 10µl added to each well. A frozen sucrose stock of *S.* Typhi attenuated strain M01ZH09 sucrose stock was thawed, washed twice with 1ml PBS then suspended in 0.3ml PBS. 2 µL 100µM BacLight Red Bacterial Stain was added to the bacterial suspension, followed by a 15 minute incubation. Bacteria were then washed with PBS, suspended in RPMI and added to each well at an MOI of 100 in triplicate. RPMI only was added to controls. The plate was then centrifuged at 500G for 3 minutes. After 2.5 hours, the plate was once again centrifuged at 500G for 3min, and the cells resuspended in 100µl PBS. 100ul of 4% formaldehyde solution was added to each well. Cells were incubated at room temperature in the dark for 30 minutes, then pelleted and resuspended in PBS. Flow cytometry was performed using a BD LSR Fortessa, measuring the fluorescence of BacLight Red Bacterial Stain and 123 Rhodamine as markers of bacterial uptake and reactive oxygen species (ROS) respectively. Viability stains and surface markers were not included in this panel for simplicity and to minimise spill-over. Compensation was carried out using unstained cells as a negative population, stained bacteria as a BacLight Red positive population, and uninfected cells treated with 123-Dihydrorhodamine as a 123-Rhodamine positive population. Granulocytes and PBMCs were gated based on forward and side scatter (see Figure 4.3); as a distinct monocyte population was not visible cells of intermediate side scatter were avoided.

Figure 4.3: Gating strategy for measuring *S.* Typhi uptake and production of reactive oxygen species by granulocytes and PBMCs. Singlets were gated based on forward scatter height and area and live cells based on absence of viability dye staining. Granulocytes and PBMCs were gated based on forward scatter (FSC) and side scatter (SSC).



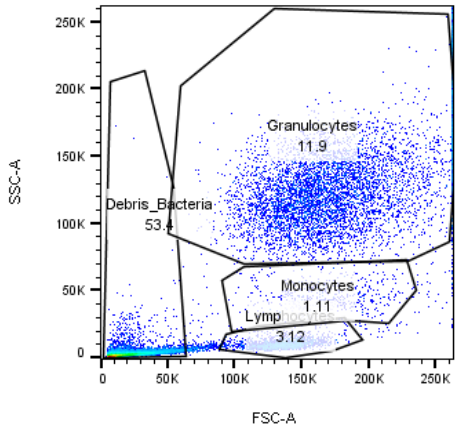
To assess the dose-dependence of uptake, white blood cells from one healthy donor were isolated by dilution 1:10 in ACK Lysing buffer, followed by seven minutes incubation at room temperature, then centrifugation at 500G for 5 minutes at room temperature. 123-Dihydrorhodamine was diluted 1:10 in PBS and 10µl added to each well. *S.* Typhi attenuated strain M01ZH09 was prepared and stained as above. Infection took place as above in triplicate for media-only controls, and in singlicate for each of MOI 10, 30 and 100. The plate was then centrifuged at 500G for 3 minutes. After 1.5 hours, the plate was once again centrifuged at 500G for 3 minutes, and the cells resuspended in 100µl PBS. 100ul of 4% formaldehyde solution was added to each well. Cells were incubated at room temperature in the dark for 30 minutes, then pelleted and resuspended in PBS. Flow cytometry was carried out as above.

4.2.13 Uptake and production of reactive oxygen species following exposure to different *Salmonella* strains

As in 3.2.7, frozen glycerol stocks of *S.* Typhi Quailes strain, isogenic toxin-negative *S.* Typhi SB6000 strain, *S.* Paratyphi A NVGH308 strain, attenuated *S.* Typhi Ty2 strain derivatives CVD 908 and CVD 909 (Tacket and Levine 2007), *S.* Typhimurium LT2 strain, and constitutive Vi^+ *S.*

Typhimurium were used to inoculate 5ml LB broth and incubated at 37°C overnight. Two further cultures of *S.* Typhi Quailes strain took place under high (0.3M) and low (9mM) NaCl conditions. Overnight cultures were diluted to a concentration of 5 x 10^8 CFU/ml, added to an equal volume of 50% glycerol in H_2O, and frozen. 5ml heparinised blood from one healthy human volunteer was incubated at room temperature for 15 minutes in three volumes of RBC lysis solution (Qiagen). White blood cells were centrifuged at 500g for 5 minutes, and washed with a further three volumes of RBC lysis solution then three volumes autoMACS Running Buffer. Cells were resuspended in RPMI with 10% FBS and seeded in a 96 well plate at a density of 100,000 cells per well. 123-Dihydrorhodamine was diluted 1:10 in PBS and 10µl added to each well. Bacterial stocks were simultaneously thawed, with one vial of *S.* Typhimurium LT2 strain heat-killed by a 20 minute incubation at 65°C. Bacteria were washed twice with RPMI and resuspended in 0.3ml PBS. 2 µL 100µM BacLight Red Bacterial Stain was added to the bacterial suspension, followed by a 15 minute incubation. Bacteria were then washed with PBS, suspended in RPMI and cells inoculated at an MOI of 10 or with RPMI as a negative control. At 2 or 4 hours post-infection cells were centrifuged at 400G for 4 minutes, and the cells resuspended in 100µl PBS. 100ul of 4% formaldehyde solution was added to each well. Cells were incubated at room temperature in the dark for 30 minutes, then at 4°C overnight. As in 4.2.12, flow cytometry was performed using a BD LSR Fortessa, measuring the fluorescence of BacLight Red Bacterial Stain and 123 Rhodamine as markers of bacterial uptake and ROS respectively. Compensation was carried out using unstained cells as a negative population, stained bacteria as a BacLight Red positive population, and uninfected cells treated with 123-Dihydrorhodamine as a 123-Rhodamine positive population. As a distinct monocyte population was visible, granulocytes, lymphocytes and monocytes were gated by forward and side scatter in FlowJo (see Figure 4.4).

Figure 4.4: Gating strategy based on forward and side scatter for granulocytes, monocytes and lymphocytes.



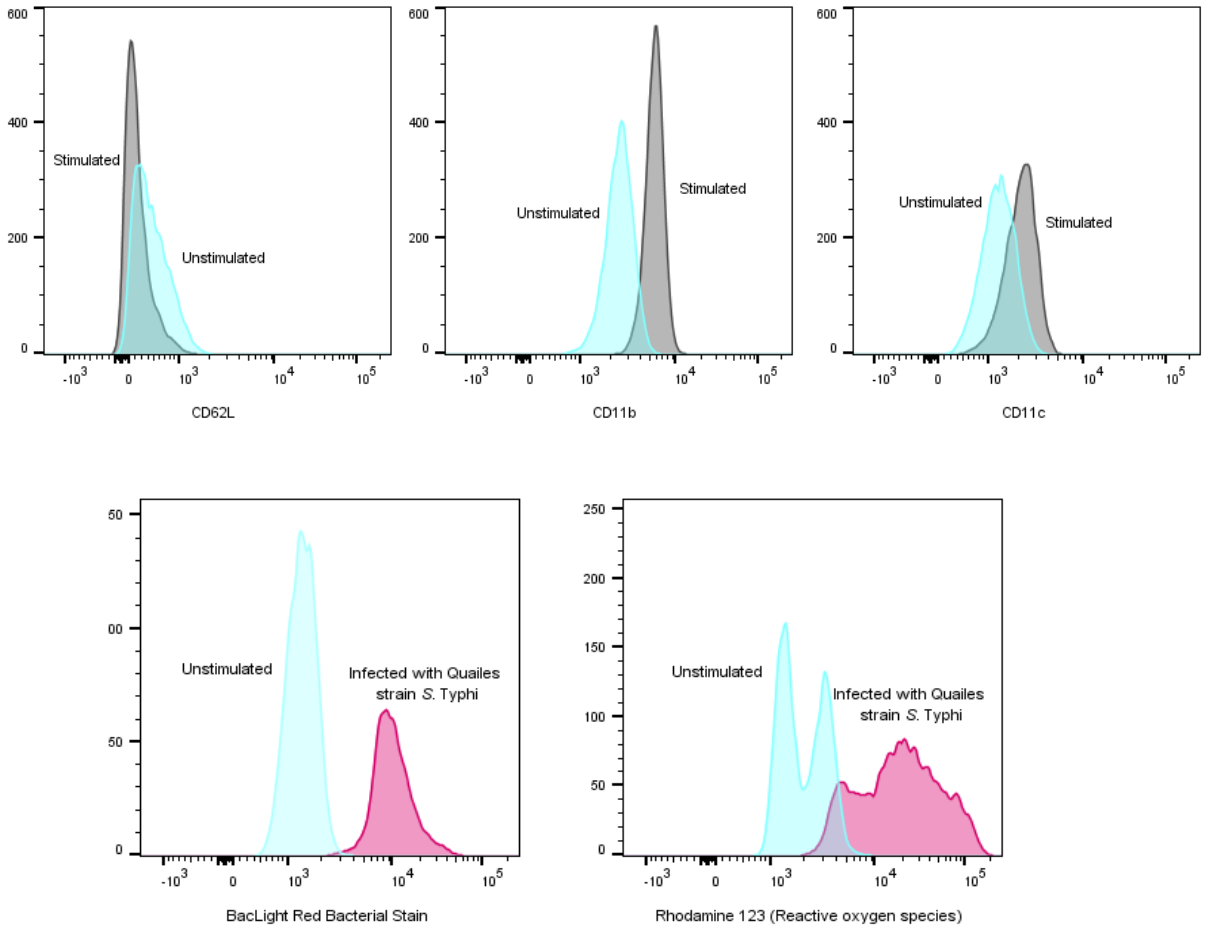
4.2.14 Statistical analysis of flow cytometry data

In order to compare levels of neutrophil surface activation markers in different conditions, the median fluorescence intensities of CD11b, CD11c and CD62L (see Figure 4.5a for distributions of fluorescence intensity) for gated granulocytes were exported from FlowJo. As batch effects in surface staining at different timepoints confounded median fluorescence intensities for *ex vivo* flow cytometry on participants from the typhoid toxin study, statistical comparisons were only made between challenged and unchallenged controls at the same timepoint (Mann-Whitney-Wilcoxon test).

For *in vitro* stimulations, a t-test was used to compare each condition with the unstimulated control. In order to assess whether there was detectable uptake of bacteria and an increase in reactive oxygen species following incubation with *S.* Typhi, the median fluorescence intensities of BacLight red and Rhodamine 123 for granulocytes and PBMCs were exported from FlowJo (see Figure 4.5b for the distributions of fluorescence intensity). A t-test was used to compare each condition with the non-infected control. To compare uptake and production of ROS in response to different bacterial strains, the median fluorescence intensities of BacLight red and Rhodamine 123 for granulocytes, monocytes, lymphocytes and free bacteria were exported from FlowJo. Due to differences in the intensity of BacLight Red staining for different bacterial strains, to calculate bacterial uptake the median fluorescence intensity of leukocytes was

normalised to the median fluorescence intensity of free stained bacteria for each strain. As granulocytes, monocytes and lymphocytes had differing levels of background fluorescence, MFIs were also normalised to that of unstimulated cells for each cell type. Normalised MFIs were then compared between bacterial strains using a t-test, focusing on comparisons of biological interest.

Figure 4.5a: Distribution of the fluorescence intensities of CD62L, CD11b and CD11c on the surface of neutrophils, unstimulated (shown in blue) or stimulated for four hours with 100ng/ml *S.* Typhi LPS (shown in grey).
b. Distribution of the fluorescence intensities of BacLight Red and Rhodamine 123 for granulocytes in the absence (shown in blue) and presence (shown in pink) of MOI 10 Quailes strain *S.* Typhi



4.2.15 Intracellular *S.* Typhi survival

15ml blood was collected from each of three healthy donors in an EDTA vacutainer, and layered onto an equal volume of Polymorphprep™ (Alere Technologies) then centrifuged at 500G for 35 minutes at room temperature with the brake off. The granulocyte and PBMC bands were transferred to separate falcon tubes and topped up to 50ml with PBS. Cells were centrifuged at 250G for 10 minutes then resuspended in 5ml RBC lysis solution for 5 minutes. The tube was once more topped up to 50ml with PBS, centrifuged at 250G for 10 minutes, and the pellets resuspended in 4ml PBS for cell counting. CD14 microbeads (Miltenyi Biotec) were diluted 1 in 5 in autoMACs running buffer and PBMCs suspended in 300µl diluted beads. After 15 minutes at 4°C 6ml autoMACS running buffer was added, and the PBMCs centrifuged for 10 minutes at 250G. PBMCs were then resuspended in 500ul running buffer, and the $CD14^+$ fraction (monocytes) and $CD14^-$ fraction (lymphocytes) separated using an autoMACS Pro Separator. Monocytes, lymphocytes and granulocytes were counted using a haemocytometer, suspended in RPMI and seeded in duplicate (two wells per donor per cell type) in a 96 well plate at a density of 100,000. Purity can be found in Table 3.2.

Frozen stocks of 10^7 CFU/ml Quailes strain *S.* Typhi were thawed and washed twice with RPMI. Cells were inoculated with *S.* Typhi at an MOI of 10. After 50 minutes, gentamycin was added to a final concentration of 200ug/ml. At one and four hours post-inoculation, the plate was centrifuged at 400G for 4 minutes, cells washed with PBS, and centrifuged again. Cells were then resuspended in 50ul 0.1% Triton X-100. After two minutes at room temperature the lysate was serially diluted in PBS and plated onto tryptone soya agar. The following day colonies in the undiluted streak were counted and averaged between replicates. A t-test was used to assess differences in CFU between the cell subsets at each timepoint.

4.2.16 Changes in gene expression following *in vitro* Hep2 and THP-1 stimulation

Publicly available raw data were downloaded from the ArrayExpress database (accession number E-MTAB-2395) (Schreiber et al. 2015). Briefly, Hep2 and THP-1 cells had been infected for one hour with *S.* Typhi Ty2 derivative BRD948 at an MOI of 10 before RNA extraction using an RNeasy Mini Kit and transcriptomic profiling using Illumina Human HT-12_V4 Beadchips. The microarray data were quantile normalised, reannotated and collapsed to genes as in 4.2.3, and samples limited to unstimulated controls and those infected with bacteria expressing *Hd*-type flagella. Genes were filtered to those retained for differential gene expression analysis in the human challenge discovery cohort. A linear regression model was then fitted to the microarray data using the limma package.

4.2.17 Changes in cytokine gene expression by different tissues in response to *Salmonella* infection

Publicly available raw data were downloaded from the Gene expression omnibus (series GSE72046) for immunocompetent mice infected for 60 days with *S.* Typhimurium possessing either an active or inactive typhoid toxin (strains MC71-TT and MC71-dcdtB respectively)(Del Bel Belluz et al. 2016). Data were normalised, background corrected, annotated using the pd.mogene.2.0.st package, and collapsed to genes using WGCNA. Limma was used to identify differentially expressed genes between uninfected and infected mice for each tissue assayed. Differential expression analysis results were downloaded from the Gene expression omnibus (series GSE113333) for human terminal ileum biopsies infected for two hours with *S.* Typhi strain Ty2.

4.2.18 Differences in cytokine gene expression in whole blood and intestinal biopsies from patients with inflammatory bowel disease (IBD)

Publicly available raw data was downloaded from the Gene expression omnibus (series GSE119600, GSE102133, GSE134025). GSE119600 contained whole blood transcriptomes

characterised by HT-12 v4 Expression BeadChip from 95 Crohn's disease patients, 93 Ulcerative Colitis patients and 47 healthy controls (Ostrowski et al. 2019). Most patients were given mesalazine prior to inclusion, but had not yet started additional treatment. GSE102133 comprised of ileal mucosal biopsies profiled by Affymetrix Human Gene 1.0 ST Array, from 65 Crohn's disease patients at a variety of disease stages (newly diagnosed, late stage, post-operative, post-operative recurrent) and 12 healthy control biopsies (Verstockt et al. 2019). GSE134025 contained the transcriptomes of three colonic mucosal biopsies from ulcerative colitis patients and three from healthy controls, profiled by Agilent-067406 Human CBC lncRNA + mRNA microarray V4.0. Data was normalised, background corrected, and collapsed to genes using WGCNA. Limma was used to identify differentially expressed genes between IBD patients and healthy controls.

4.3 Results

4.3.1 The human blood transcriptome is perturbed 12 hours after *S.* Typhi challenge

To investigate early responses to human *S.* Typhi challenge, changes in gene expression were measured at baseline, 12 hours and 24 hours post-challenge using Illumina HT-12v4 bead-arrays. As challenge typically took place in the morning, the data was integrated with a publicly available dataset here referred to as the "unchallenged control" dataset to allow us to assess the contribution of circadian rhythms to changes in gene expression.

12 hours post-baseline, 2964 and 16 genes were differentially expressed in the challenged and unchallenged cohorts respectively (FDR < 0.05, Benjamin-Hochberg correction, Figure 4.6). Only a small subset of genes differentially expressed post-challenge were also significantly changed in the unchallenged cohort (8/2964, 0.3%). However, genes such as *DDIT4-AS1*, *FKBP5*, and *KLF9* were highly significant in both cohorts, suggesting circadian rhythms are a

potential confounder. To eliminate the effect of circadian rhythms, including any genes which may be circadian regulated but did not reach significance in the unchallenged cohort due to small sample size, a "difference of differences" contrast was used to identify changes in gene expression likely attributable to typhoid challenge alone. This contrast left 216 differentially expressed genes (Table 4.3). Despite the approach used to exclude the circadian effects, overall the expression of the 216 challenge-specific genes at 24 hours post-challenge appears to be returning to baseline (Figure 4.7).

Figure 4.6: Volcano plots showing the $\log_2$(Fold Change) in gene expression against the -log10(Benjamin-Hochberg adjusted p-value) 12 hours post-baseline for the discovery cohort (typhoid dose finding study and typhoid oral vaccine trial, in addition to publicly available unchallenged controls) relative to baseline. RNA expression was characterised by microarray, background corrected, normalised and batch corrected, and differential expression assessed using the limma R package, using participant ID as a blocking variable and dose and batch as covariates. Green points represent genes differentially expressed (FDR<0.05) in an unchallenged cohort, and are highlighted in each plot. Pink points represent genes differentially expressed in participants challenged with S. Typhi, and orange points genes differentially expressed after challenge following adjustment for unchallenged controls. Lighter coloured points indicate genes with a p-value <0.05 before but not after adjusting for multiple testing. The size of each point reflects average gene abundance ($\log_2$(Probe Intensity)).

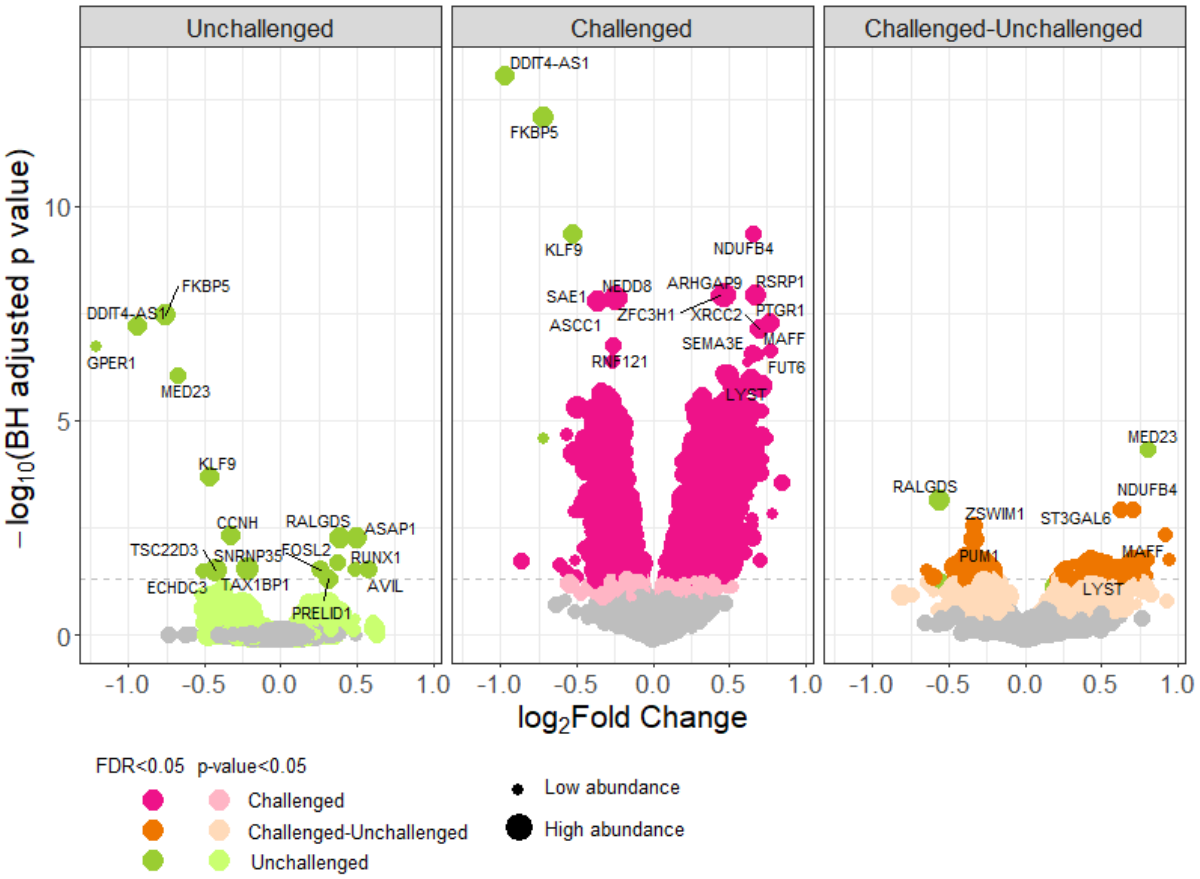
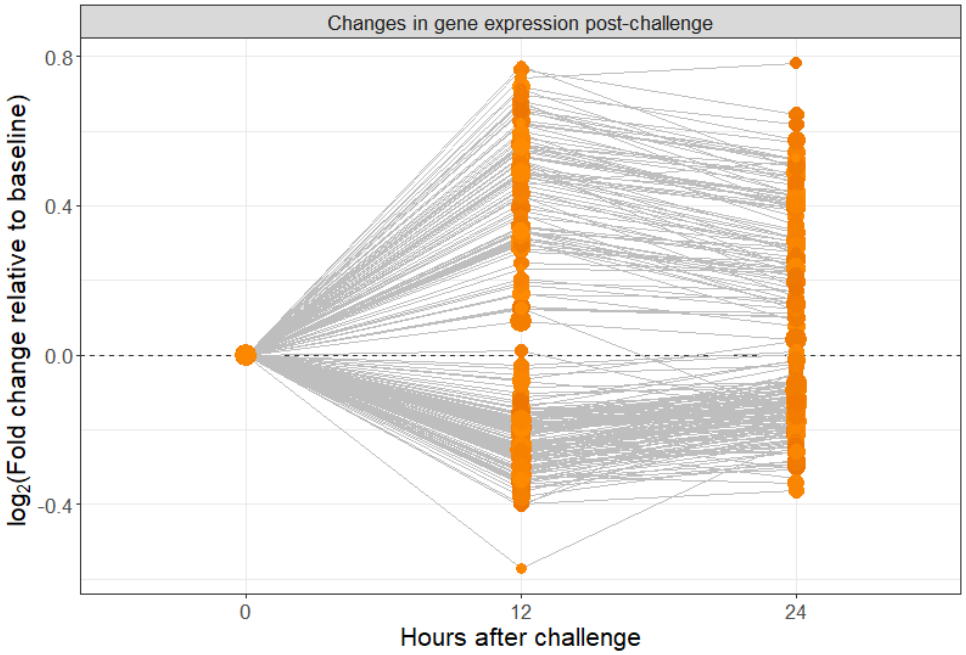


Table 4.3: Top 10 most statistically significant challenge-specific genes as assessed using the Limma R package, and their functions according to Entrez Gene.

HGNC Symbol	Function	Log_2Fold Change		FDR adjusted p-value
		Challenged	Unchallenged	
MED23	Required for SP1 activation	0.12	-0.68	0.00005
RALGDS	Guanine nucleotide dissociation stimulator	-0.18	0.39	0.0007
ST3GAL6	Sialyltransferase involved in formation of selectin ligands and CD15	0.38	-0.25	0.001
NDUFB4	Subunit of NADH:ubiquinone oxidoreductase	0.65	-0.05	0.001
ZSWIM1	Unknown	-0.16	0.18	0.003
MAFF	Basic leucine zipper transcription factor involved in stress response	0.78	-0.14	0.005
PUM1	Translational regulator	-0.21	0.13	0.006
TRAF3IP2	NF-kB activator	-0.29	0.11	0.01
ALKBH5	RNA demethylase	-0.24	0.08	0.02
PTGR1	Inactivation of chemotactic factor leukotriene B4	0.78	-0.02	0.02

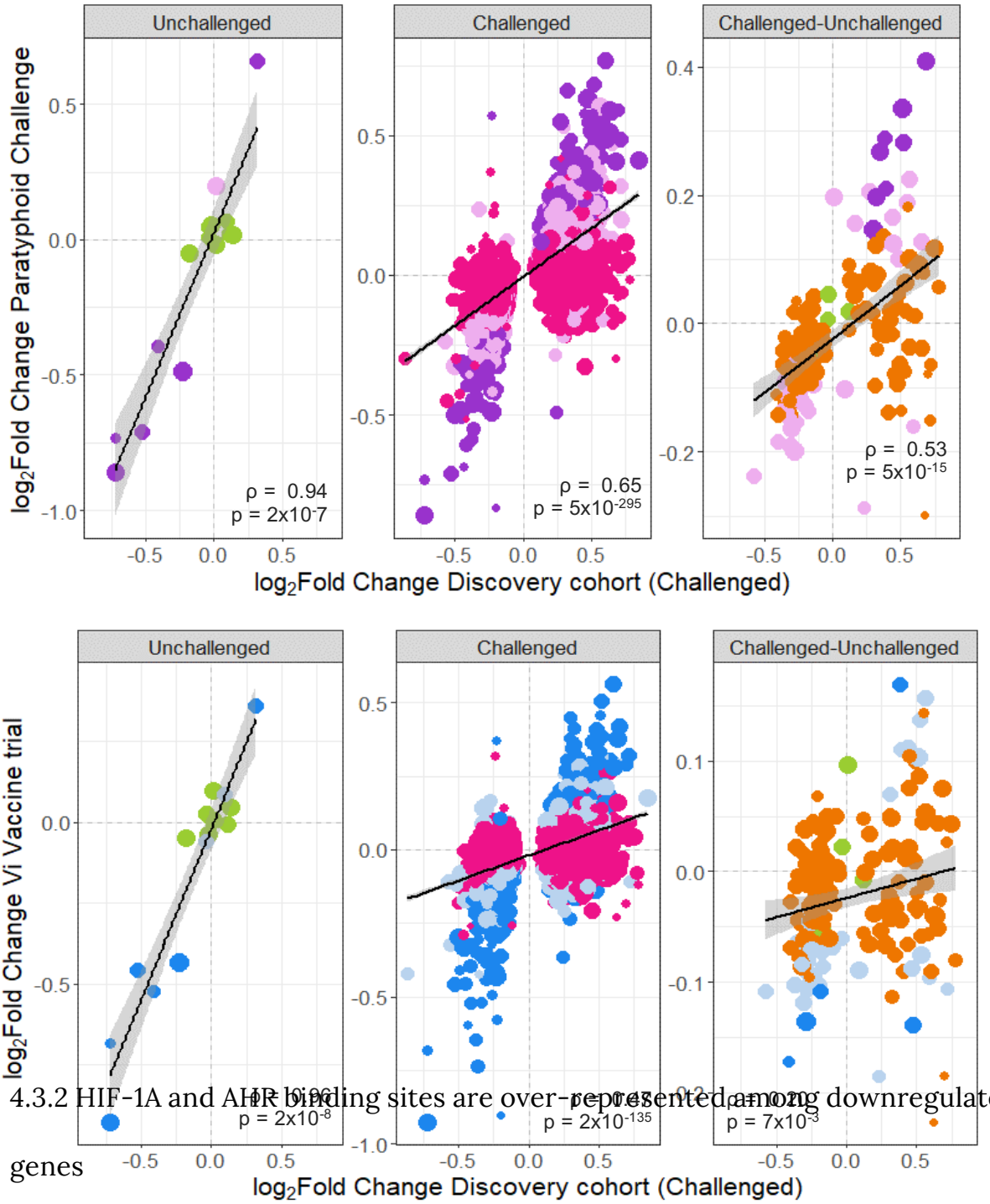
Figure 4.7: Changes in gene expression relative to baseline over time over the first 24 hours post-challenge, in the genes comprising the early typhoid-specific response. Grey lines join the same gene at different time points.



To assess the reproducibility of changes in gene expression, the log_2fold changes of differentially expressed genes identified in analysis of the discovery cohort were compared

with those from two independent cohorts: a paratyphoid challenge study and a typhoid polysaccharide (Vi) vaccine trial. Circadian genes, those which were differentially expressed in the unchallenged cohort, were tightly correlated between datasets (Figure 4.8 left panel; $\rho > 0.9$ and $p < 2 \times 10^{-7}$ for both comparisons), whereas challenge-specific genes were less markedly but still significantly correlated (Figure 4.4 right panel, Table S4.3; $\rho > 0.2$ and $p < 7 \times 10^{-3}$ for both comparisons).

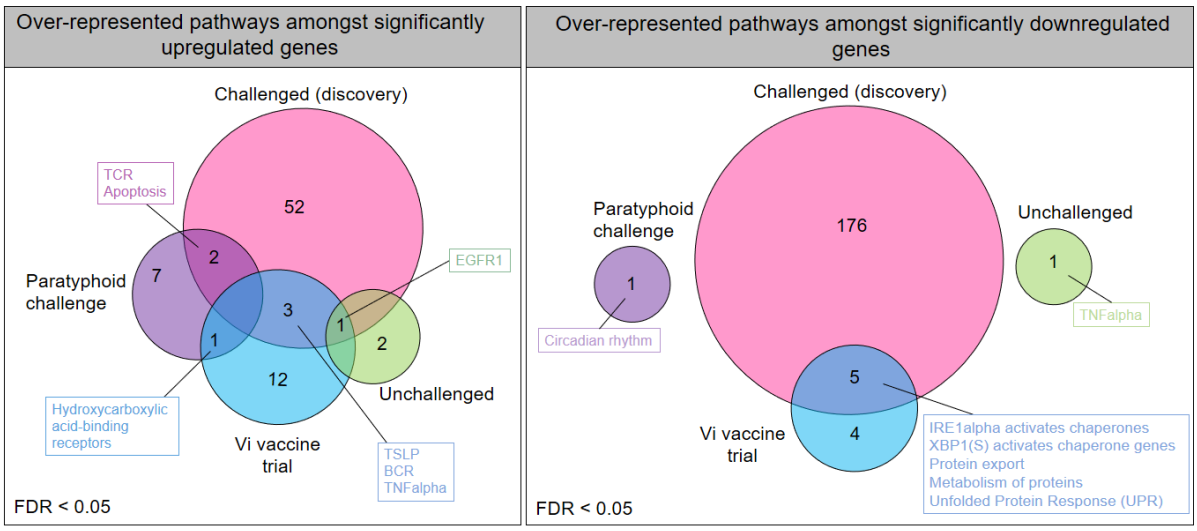
Figure 4.8: Correlation in log_2fold change 12 hours post-challenge between the challenged discovery cohort and two validation cohorts, which were derived from the typhoid Vi vaccine and paratyphoid dose finding studies and both profiled by RNA-sequencing. Genes were subset to those differentially expressed (FDR<0.05) in the discovery cohort (n = 16, 2964 and 216 genes for the unchallenged, challenged and challenged-unchallenged comparisons respectively). Those which were also differentially expressed in the validation cohorts are highlighted in purple (Paratyphoid challenge) or blue (Vi vaccine trial). A linear regression line with 95% shaded confidence intervals is shown in black. The size of each point reflects average gene abundance (log_2(counts per million)) in the validation cohort. The Pearson correlation coefficient (R) and p-value for a test of association between the two variables is shown.



4.3.2 HIF-1A and AHR binding sites are over-represented among downregulated genes

Pathway analysis was used to identify pathways over-represented amongst significantly up- and down-regulated genes 12 hours post-challenge (Figure S4.5). No significantly over-represented pathways (FDR < 0.05, hypergeometric test) were shared between all three challenged cohorts (Figure 4.9).

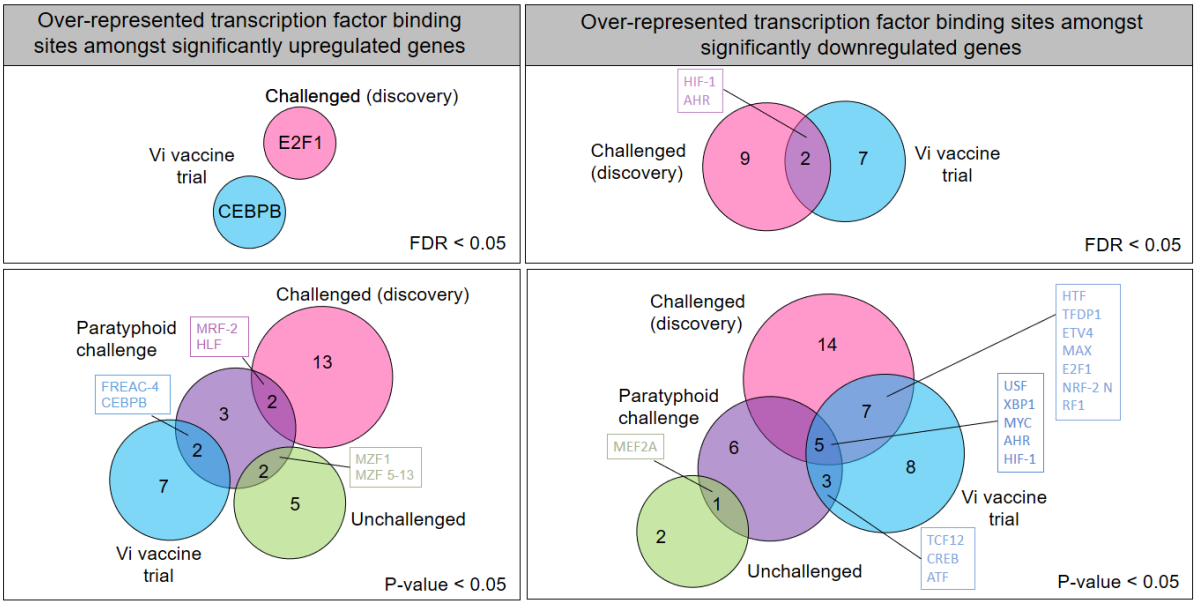
Figure 4.9: Overlap in significantly over-represented (FDR < 0.05) pathways amongst up- and downregulated genes between cohorts. Pathway analysis was carried out using InnateDB, with pathways over-represented amongst sets of genes identified using a hypergeometric test, and p-values adjusted for multiple testing using a Benjamin-Hochberg correction. Pathways from the KEGG, REACTOME, NETPATH, PID NCI, PID BIOCARTA and INOH databases were included.



Transcription factor binding site over-representation analysis was subsequently used to identify transcription factors that may be controlling changes in gene expression post-challenge. Binding sites for HIF-1A and AHR were identified as being over-represented amongst genes downregulated in the challenged discovery cohort and Vi vaccine trial (FDR < 0.05, hypergeometric test, Figure 4.10, upper panel). Relaxing the significance threshold to an unadjusted p value < 0.05, it was found that binding sites for HIF-1A and AHR were also over-represented amongst genes downregulated after *S.* Paratyphi A challenge (Figure 4.10, lower panel). Although HIF-1A and AHR binding sites were consistently more significantly over-represented amongst the downregulated than the upregulated genes in each cohort (Figure S4.6) the expression of *HIF1A* and *AHR* themselves did not follow a consistent pattern (Figure S4.7). Pathway analysis was then carried out on HIF-1A and AHR target genes which were

specifically downregulated in response to challenge (Table S4.4), and included pathways related antigen and protein processing.

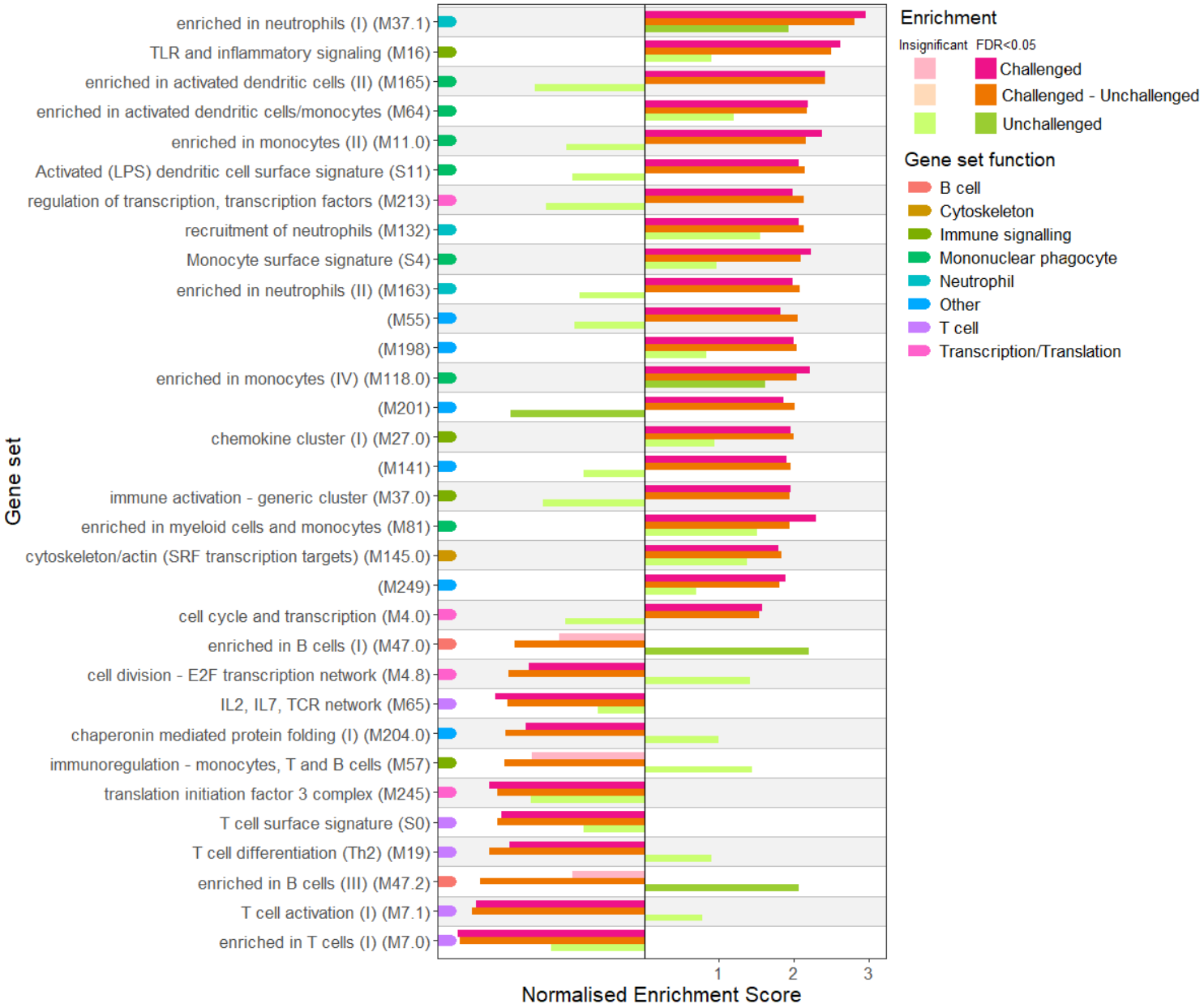
Figure 4.10: Overlap in over-represented transcription factor binding sites (top panel: FDR < 0.05, bottom panel: p < 0.05) amongst genes up- and downregulated between cohorts. Transcription factor binding site over-representation analysis was carried out using InnateDB, with binding sites over-represented amongst sets of genes identified using a hypergeometric test, and p-values adjusted for multiple testing using a Benjamin-Hochberg correction. Transcription factor binding sites were all derived from the cisRED database.



4.3.3 Gene sets relating to innate immunity are over-represented post-challenge

Gene set enrichment analysis was used to identify whether pre-defined blood transcriptional modules were changed after challenge. 12 hours post-challenge, 75/346 gene sets were significantly (FDR < 0.05) over or under-represented relative to baseline in the discovery cohort, compared with 21/346 in the unchallenged cohort. 63/346 gene sets were significantly enriched in the challenge-response compared with the control response (Figure 4.11). Gene sets relating to neutrophils, inflammatory signalling, monocytes and dendritic cells tended to be over-represented, while B and T cell related gene sets were under-represented.

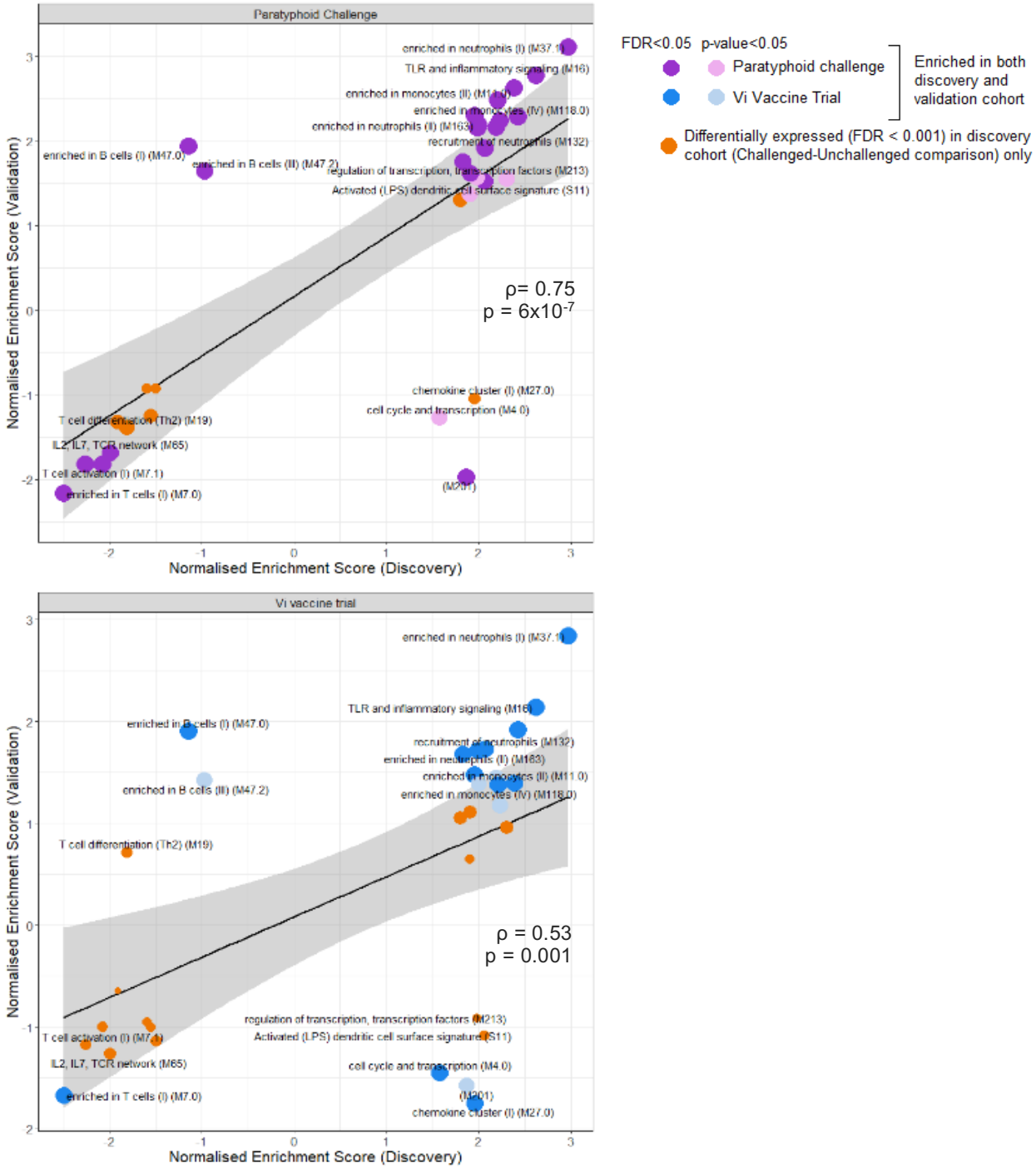
Figure 4.11: Normalised enrichment scores of gene sets significantly over- or under-represented in the early post-challenge response relative to unchallenged controls (FDR < 0.001). Normalised enrichment scores for the challenged cohort only are shown in pink, unchallenged in green, and the challenged – unchallenged comparison in orange. Bars are filled in a lighter colour if they did not reach significance (FDR < 0.05) in the challenged or unchallenged cohort alone. Modules M55, M198, M201, M141 and M249 have not been annotated with a function.



As above, to assess reproducibility the normalised enrichment scores of gene sets enriched in the challenge-specific response were compared with those from the independent cohorts (Figure 4.12). Correlation was high (ρ = 0.75, p = 6×10^{-7}, and ρ = 0.53, p = 0.001; Pearson

correlation) for the paratyphoid challenge and Vi vaccine trial cohorts respectively) with a consistent over-representation of innate immune related gene sets. However while B cell modules M47.0 and M47.2 were under-represented in the discovery datasets (microarray), they were over-represented in both of the validation (RNA-sequencing) datasets. The converse was true of modules M201 (unassigned function), M4.0 (cell cycle) and M27.0 (chemokines).

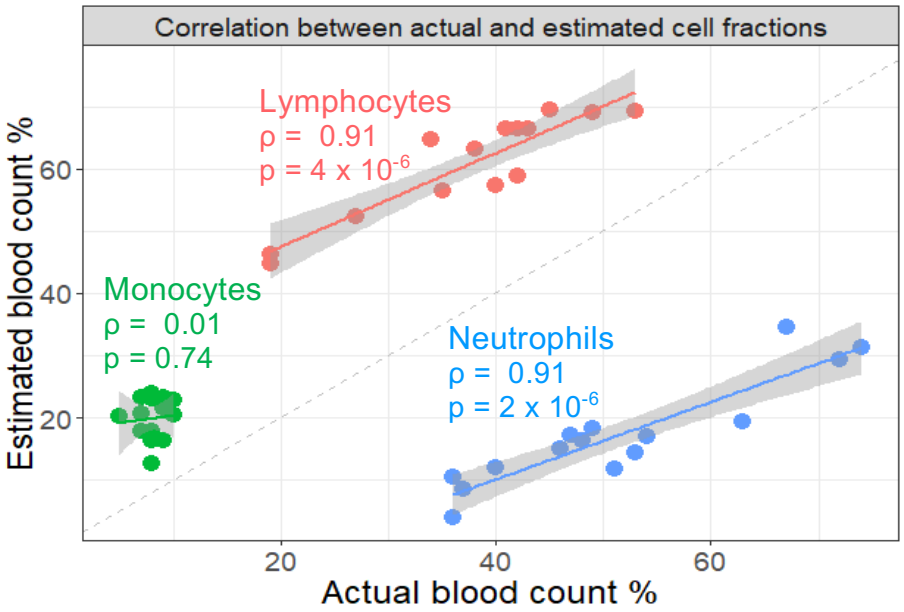
Figure 4.12: Correlation of normalised enrichment scores 12 hours post-challenge between the challenged discovery cohort and two validation cohorts. Gene sets were subset to those highly enriched (FDR<0.01) in the discovery cohort. Those which were also enriched in the validation cohorts are highlighted in purple (Paratyphoid challenge) or blue (Vi vaccine trial). A linear regression line with 95% shaded confidence intervals is shown in black. The Pearson correlation coefficient (ρ) and p-value for a test of association between the two variables is shown. Highlighted module has not been annotated with a function.



4.3.4 Deconvolution suggests an increase in neutrophil counts early after challenge

To explore the over-representation of gene sets relating to innate immune cells, CIBERSORT was used to estimate changes in immune cell fractions. For a subset of samples differential blood counts were available, and correlated with *in silico*-generated estimates of cell fraction (Figure 4.13). Despite over-estimation of the lymphocyte: neutrophil ratio, correlation between actual and estimated lymphocyte and neutrophil counts was high ($\rho = 0.91$, $p = 4 \times 10^{-6}$; Pearson correlation), validating the technique as a means to estimate changes in these fractions. Both techniques identified monocytes as being less abundant than the other fractions (<25% cells) but correlation was poor ($\rho = 0.01$, $p = 0.74$).

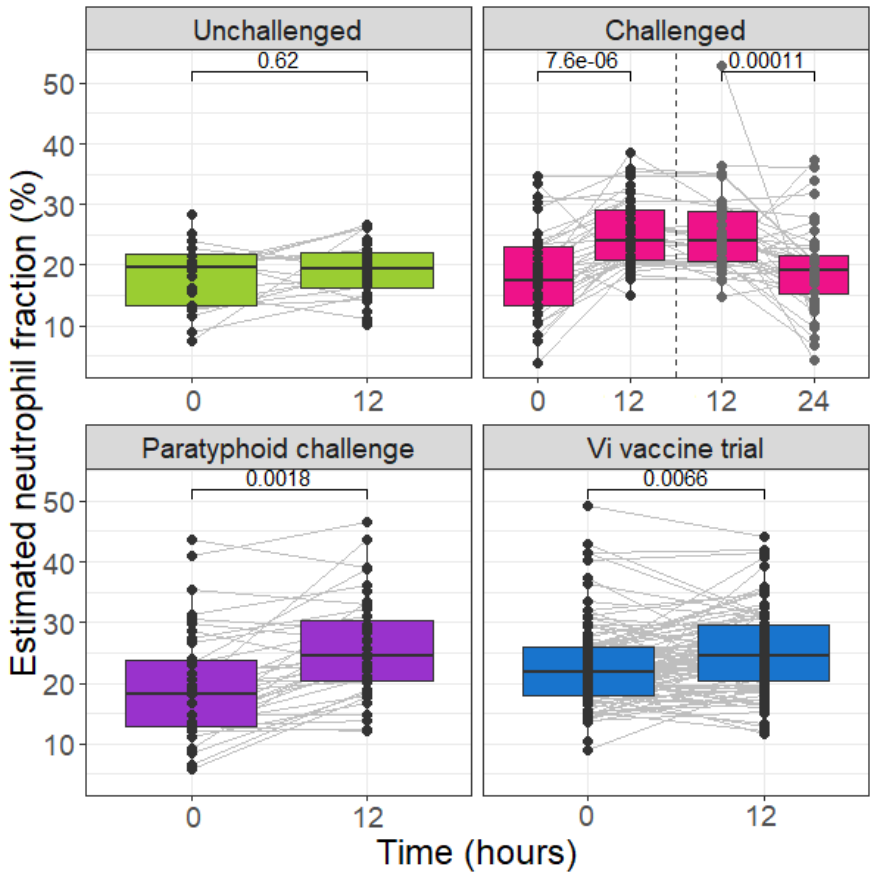
Figure 4.13: Correlation between differential blood counts and cell fractions estimated from microarray data using CIBERSORT. The Pearson correlation coefficient (ρ) and p-value for a test of association between the two variables is shown.



12 hours-post challenge, estimated neutrophil fraction was significantly raised in all challenged cohorts (Figure 4.14, $p < 0.01$ in all cohorts, Wilcoxon signed-rank test), while no change was seen in unchallenged controls ($p = 0.62$). However, 24 hours after challenge the neutrophil

fraction returned baseline levels. No significant differences in the neutrophil fraction or the magnitude of the change in this fraction between baseline and 12 hours post-challenge were observed between those who went on to develop enteric fever and those who stayed healthy.

Figure 4.14: Changes in estimated neutrophil fraction post-challenge. For the challenged discovery cohort, estimated neutrophil fractions from batches 1 and 3 (paired baseline and 12 hours samples) and batch 2 (paired 12 hour and 24 samples) are shown separately. A Wilcoxon signed-rank test was used to calculate the indicated p-values.



4.3.5 *S.* Typhi activates neutrophils and survives intracellularly

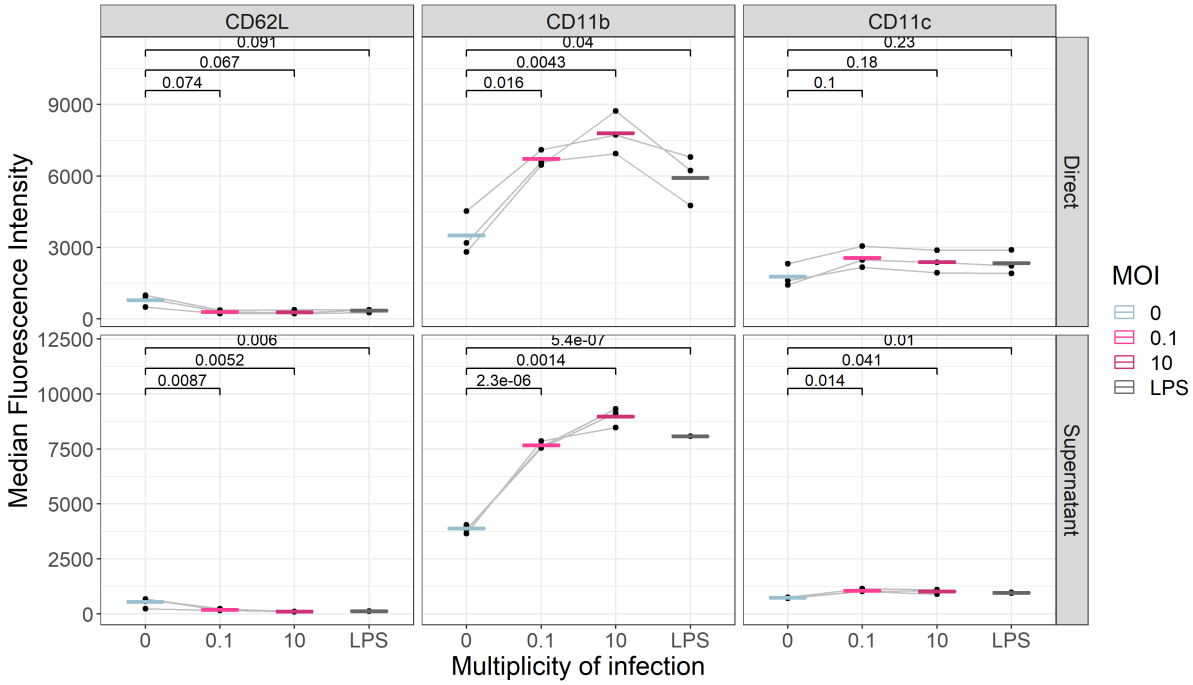
To further explore whether a rise in neutrophils would likely confer a protective or detrimental effect to the host, *S.* Typhi-neutrophil interactions were investigated through *in vitro* stimulation of white blood cells with *S.* Typhi Quailes strain. Phenotyping by flow cytometry identified surface markers CD11b and CD11c as being upregulated in response to infection, while CD62L was shed (Figure 4.15a, $p < 0.1$ for a two sample t-test between unstimulated cells and

those stimulated with an MOI of 0.1). Culturing white blood cells in filtered supernatants from infected cells also resulted in neutrophil activation (Figure 4.15b).

Figure 4.15: a. Median fluorescence intensities of activation markers on neutrophils from three healthy donors after stimulation with *S.* Typhi at a multiplicity of infection 0.1 or 10, or with *S.* Typhi LPS. Red blood cells from heparinised blood were lysed, and the white blood cells washed and seeded before stimulation with ether with *S.* Typhi at an MOI of 10 or 0.1, 100ng/ml *S.* Typhi LPS as a positive control, or RPMI as a negative control. After one hour extracellular bacteria were killed using 100μg/ml gentamycin. After a further four hours, cells were washed and stained with a viability dye and then a CD62L, CD11b, CD11c and CD16 antibody cocktail. Cells were fixed using 4% formaldehyde before determining MFIs by flow cytometry. Neutrophils were gated based on forward scatter, side scatter and CD16 expression. Lines join points from the same donor. b. Median fluorescence intensities of activation markers on neutrophils from one healthy donor after culture in supernatants from the above three donors for four hours, or stimulated with LPS. Cells were washed and stained as above. Lines join points where supernatants came from stimulation of the same donor cells. A t-test was used to calculate the indicated p-values.

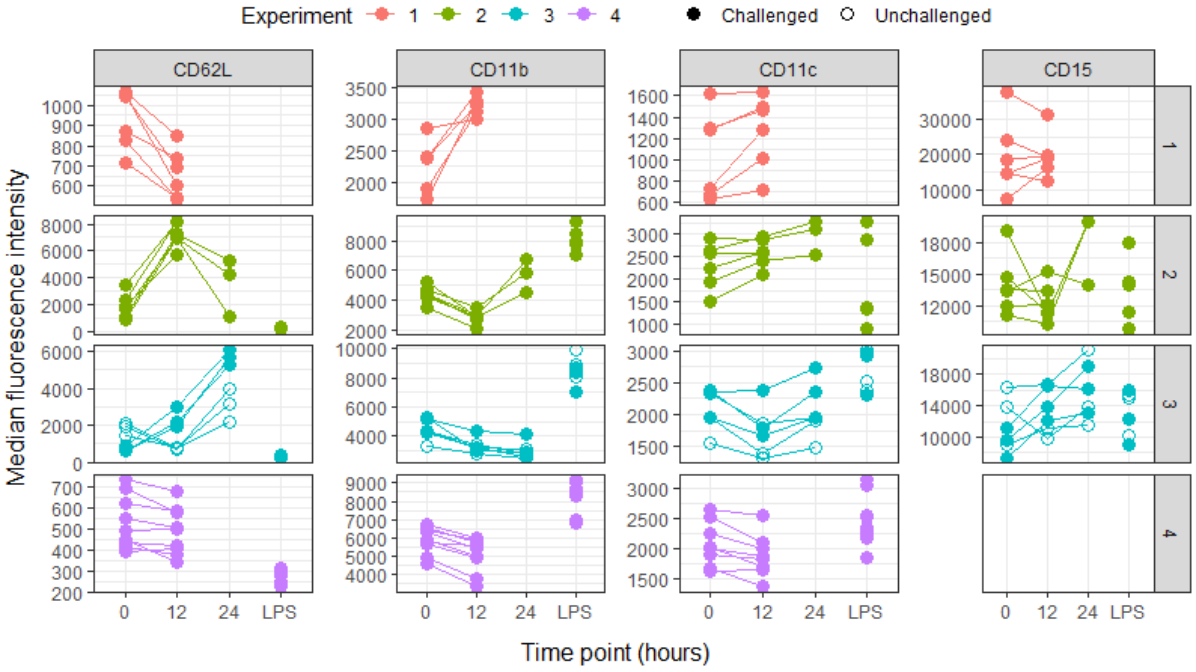
a.

b.



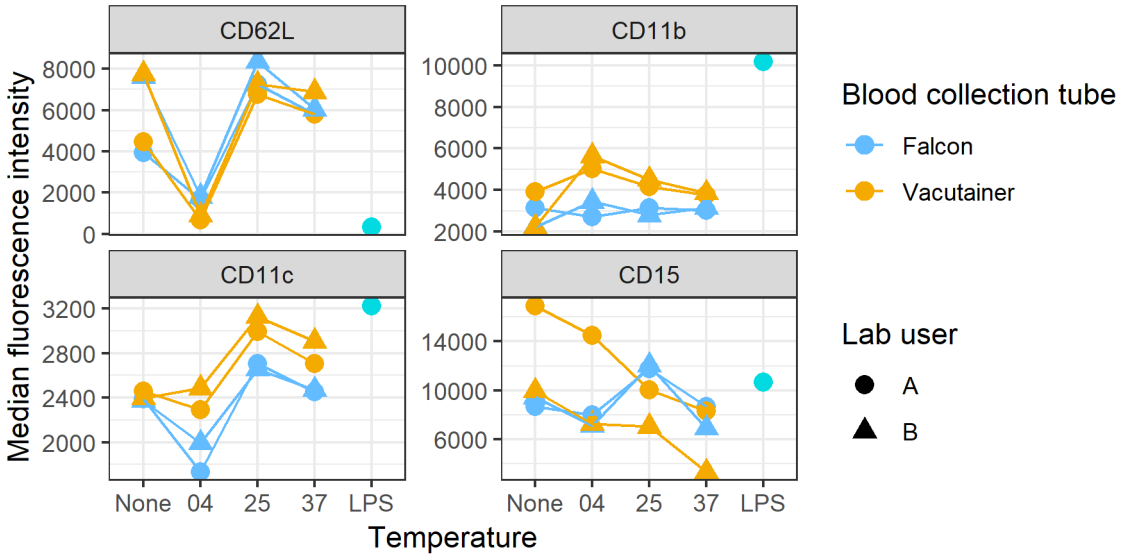
To investigate whether neutrophil activation could be detected *ex vivo* post-challenge, fresh white blood cells were isolated at baseline and post-challenge in participants taking part in the typhoid toxin study and phenotyped. There was a small trend towards higher CD62L expression 12 hours post-challenge compared with unchallenged control samples taken at the same time ($p = 0.1$, Mann-Whitney-Wilcoxon test). However, there was marked variability in neutrophil activation status between repeated experiments (Figure 4.16), possibly due to small differences in sample processing.

Figure 4.16: Median fluorescence intensities of neutrophil surface markers CD62L, CD11b, CD11c and CD15 at baseline, 12 hours post-challenge and 24 hours post-challenge, carried out on four separate dates. Blood samples were taken from participants enrolled in the typhoid toxin study. Immediately after collection at each time point, red blood cells were lysed and white blood cells washed before staining with viability dye then a CD62L, CD11b, CD11c and CD16 antibody cocktail. Cells were fixed using 4% formaldehyde before determining MFIs by flow cytometry. Neutrophils were gated based on forward scatter, side scatter and CD16 expression. Where neutrophils from unchallenged controls were phenotyped points are shown as an empty circle.



In order to address the variability in results, confounding factors which might affect the measurement of neutrophil activation were investigated, including blood collection tube type, fluorophore conjugated to the CD62L detection antibody, buffer in the staining mixture, shaking, temperature, and person carrying out the staining (Figure S4.8). On the basis that keeping blood samples on ice had the greatest noticeable effect on CD62L shedding and CD11b upregulation (Figure 4.17), in the following human typhoid challenge (Figure 4.16 experiment 4) samples were kept at room temperature throughout staining. However, neutrophils were noticeably activated even at baseline.

Figure 4.17: Median fluorescence intensities of neutrophil surface markers CD62L, CD11b, CD11c and CD15 in cells collected from one healthy donor, processed immediately, or stored for 2 hours either on ice, at room temperature or in a 37°C incubator, or stimulated with LPS. Red blood cells were then lysed and white blood cells washed before staining with viability dye then a CD62L, CD11b, CD11c and CD16 antibody cocktail. Cells were fixed using 4% formaldehyde before determining MFIs by flow cytometry. Neutrophils were gated based on forward scatter, side scatter and CD16 expression.



To investigate whether neutrophils are a likely host cell during early infection, *in vitro* infection followed by cell lysis and agar plating was used to quantify bacterial survival within different immune cells isolated from whole blood. White blood cells from healthy individuals were separated into granulocytes (typically >90% neutrophils), monocytes and lymphocytes. At one hour post-infection there was a significantly greater number of *S.* Typhi surviving within monocytes than within granulocytes ($p = 0.003$, t-test) or lymphocytes ($p = 0.01$). To assess whether this represented a lack of uptake or rapid killing by granulocytes, bacterial uptake and reactive oxygen species (ROS) were simultaneously measured by flow cytometry. A high MOI of 100 was used on the basis of a preliminary experiment showing low signal at smaller MOIs. Granulocytes took up a greater number of bacteria than PBMCs ($p = 0.01$, t-test), with even low MOIs resulting in an oxidative burst. Thus, it appears that the lower number of live bacteria in granulocytes one hour post-infection is likely due to rapid killing (Figure 4.18.a). Both uptake and induction of ROS were dose-dependent (Figure 4.18.b). However, by four hours post-

infection a sub-population of bacteria were still surviving within granulocytes, with no difference in the number of surviving bacteria between each cell type (Figure 4.19).

Figure 4.18: Median fluorescence intensities of BacLight Red Bacterial Stain, reflecting bacterial uptake, and 123-Rhodamine, reflecting the production of reactive oxygen species, in granulocytes and PBMCs infected with *S.* Typhi M01ZH09 strain. a. White blood cells from one healthy donor were isolated by red blood cell lysis using Qiagen RBC lysis buffer, washed and incubated with 123-Dihydrorhodamine before infection in triplicate at MOI 100 with *S.* Typhi attenuated strain M01ZH09 stained with BacLight Red Bacterial Stain. After 2.5 hours cells were fixed using 4% formaldehyde before determining MFIs by flow cytometry. Granulocytes and PBMCs were gated based on forward and side scatter. b. White blood cells isolated by red blood cell lysis ACK buffer, washed and incubated with 123-Dihydrorhodamine before infection in singlicate at MOIs of 10, 30 and 100 with *S.* Typhi attenuated strain M01ZH09 stained with BacLight Red Bacterial Stain. After 1.5 hours cells were fixed using 4% formaldehyde before determining MFIs by flow cytometry. Granulocytes and PBMCs were gated based on forward and side scatter. Asterisks indicate the results of a one-sample t-test ("***"=0.001, "**"=0.01, "*"=0.05).

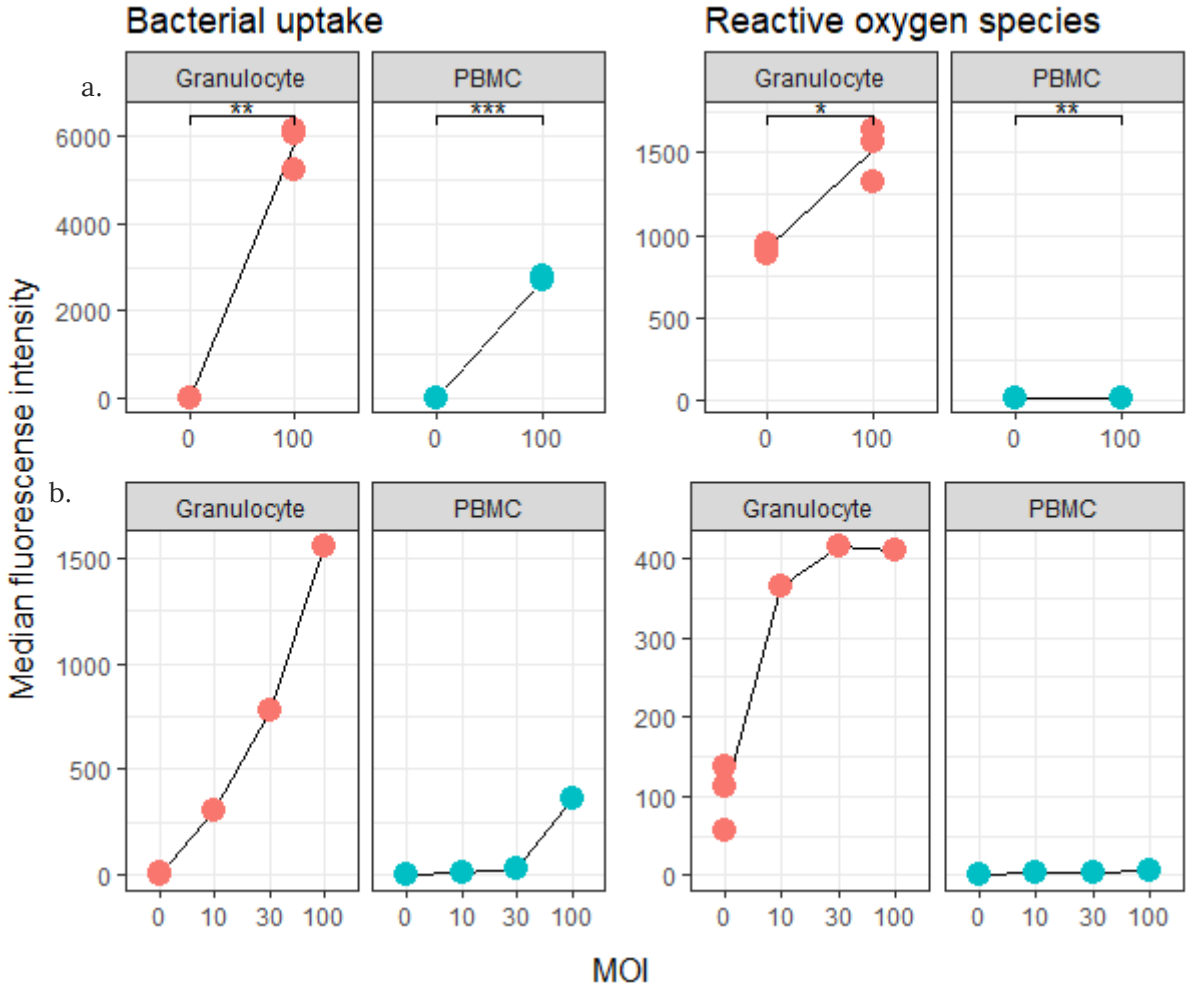
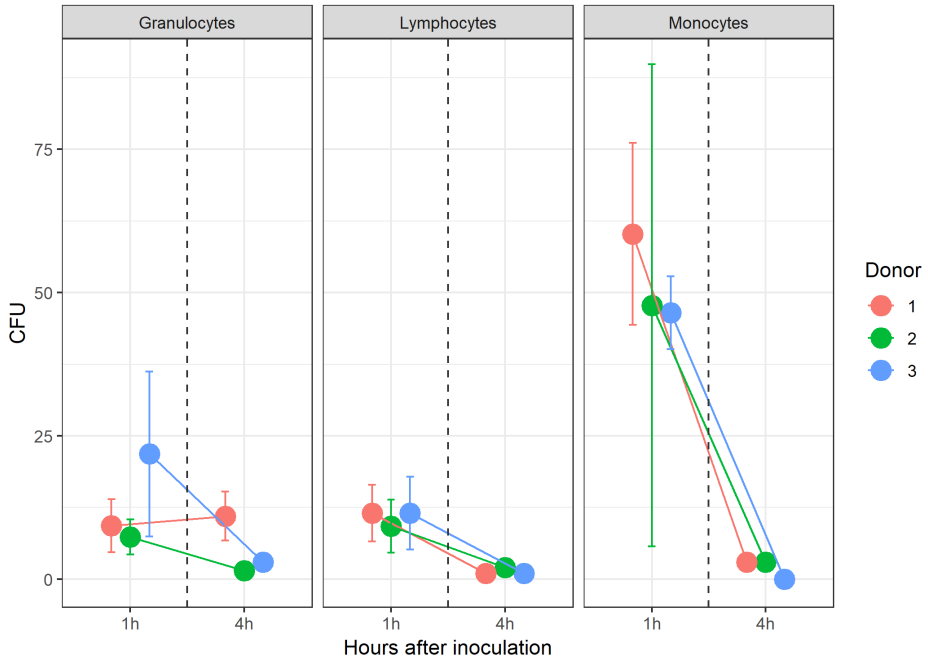
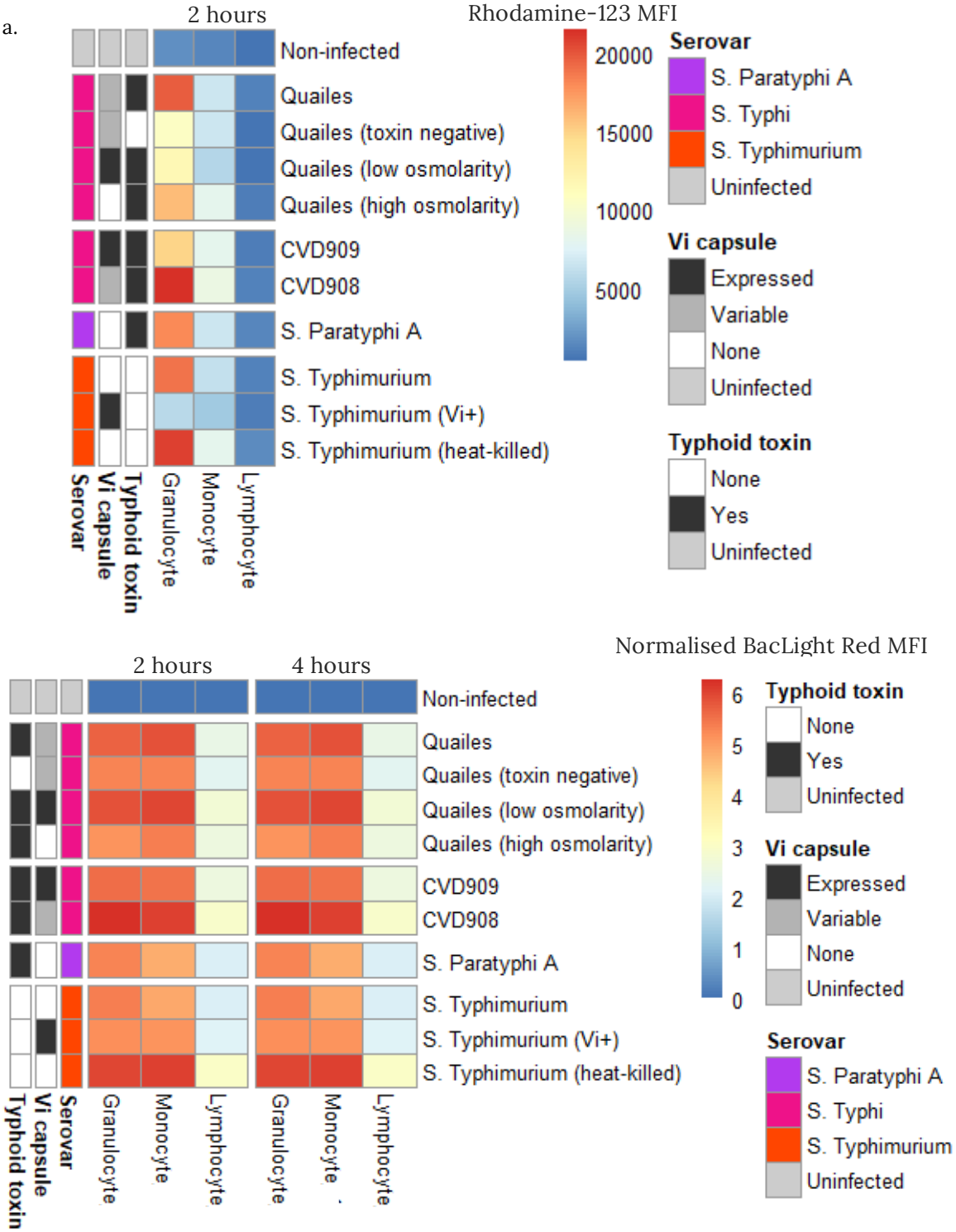


Figure 4.19: Changes in colony forming units (CFU) recovered from 10µl lysed granulocytes, lymphocytes and monocytes infected with Quailes strain *S.* Typhi at an MOI of 10, one and four hours post-inoculation. Blood was collected from three donors and granulocytes and PBMCs separated by density gradient centrifugation. PBMCs were separated into monocytes and lymphocytes by CD14 selection using an autoMACS Pro Separator. Each cell type from each donor was seeded in duplicate and infected with *S.* Typhi Quailes strain at an MOI of 10. Gentamycin was added to kill extracellular bacteria after 50 minutes. At one and four hours post-inoculation, cells were lysed by suspension in 0.1% Triton X-100, serially diluted in PBS, and plated onto tryptone soya agar. Colonies in the undiluted streak were counted the following day. Points are colour coded by healthy blood donor, with error bars indicating the standard deviation for duplicate wells independently inoculated and incubated with *S.* Typhi.



Infection of white blood cells using different strains of *Salmonella* was carried out to identify microbial factors which may affect neutrophil responses (Figure 4.20a). A strain of *S.* Typhimurium constitutively expressing the Vi capsule elicited significantly lower ROS in granulocytes than wild-type *S.* Typhimurium ($p = 0.01$, t-test). *S.* Typhi CVD 909 strain, which constitutively expresses Vi, provoked lower ROS than parent strain CVD 908 ($p = 0.1$), and *S.* Typhi Quailes in the low osmolarity conditions thought to induce Vi expression elicited lower ROS than in high osmolarity conditions. For each strain, bacterial uptake was similar between granulocytes and monocytes, but lower for lymphocytes (Figure 4.20b). At 4 hours post-infection, uptake of Quailes was greater than *S.* Paratyphi A ($p = 0.004$), *S.* Typhimurium ($p = 0.003$) or Quailes grown in high osmolarity conditions ($p = 0.04$).

Figure 4.20: a. Production of ROS (as indicated by the MFI of Rhodamine-123) by granulocytes, monocytes and lymphocytes 2 hours post-stimulation with a variety of *Salmonella* strains, the properties of which are indicated in the side bar. White blood cells were isolated from one healthy donor and incubated with 123-Dihydrorhodamine. Bacterial stocks of *S.* Typhi Quailes strain (cultured under usual, high and low osmolarity conditions), isogenic toxin-negative *S.* Typhi SB6000 strain, *S.* Paratyphi A NVGH308 strain, attenuated *S.* Typhi Ty2 strain derivatives CVD 908 and CVD 909, *S.* Typhimurium LT2 strain and constitutive Vi$^+$ *S.* Typhimurium were simultaneously thawed, with one vial of *S.* Typhimurium LT2 strain heat-killed by a 20 minute incubation at 65°C. Bacteria were washed twice with RPMI and stained with BacLight Red Bacterial Stain. Bacteria were then washed with PBS, suspended in RPMI and cells inoculated at an MOI of 10 or with RPMI as a negative control in duplicate. At 2 hours post-stimulation cells were fixed with formaldehyde, and MFIs collected by flow cytometry. Granulocytes, monocytes and lymphocytes were gated using forward and side scatter.
b. Bacterial uptake (as indicated by the MFI of BacLight Red, normalised to the MFI of free bacteria for each strain and the MFI of unstimulated granulocytes, monocytes and lymphocytes) species by granulocytes, monocytes and lymphocytes 2 and 4 hours post-stimulation with a variety of *Salmonella* strains, the properties of which are indicated in the side bar. Cells and bacteria were treated as above.



4.3.6 There is no early difference at the transcriptional level between those who go on to develop enteric fever and those who do not

Changes in gene expression between the baseline and 12 hour time-point correlated well between those who went on to develop enteric fever and those who did not, with no genes significantly differentially changed between the two groups ($\rho = 0.69$, $p < 1 \times 10^{-15}$, Figure 4.21). Although participants from the same outcome group clustered together in a principal component analysis based on the top 10 genes most differentially expressed between the two groups, these same genes could not predict outcome in the validation cohorts (Figure 4.22). Likewise, although several gene sets were significantly different (FDR < 0.05) between those who did and do not develop typhoid fever in the discovery cohort, these could not be reproduced in the validation cohorts (Figure S4.9). However, in all cohorts there was a trend towards those who remained healthy having a greater magnitude of response (Figure 4.23).

Figure 4.21: Correlation in $\log_2$(Fold change gene expression between baseline and 12h) between those who remained healthy following challenge and those who went on to develop typhoid fever. Points are coloured according to whether the gene reached significance (FDR < 0.05) in each of the groups. A linear regression line with 95% shaded confidence intervals is shown in blue.

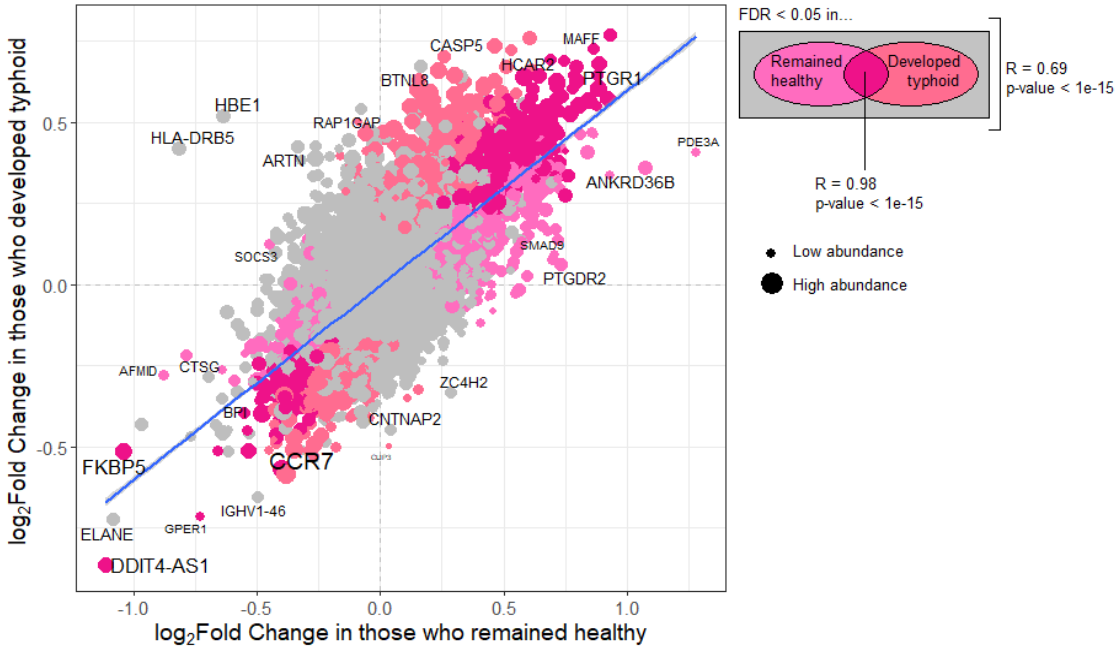


Figure 4.22: Principal components based on the $\log_2$(fold change in expression 12 hours post-challenge relative to baseline) of the ten genes most differentially expressed between those who did and did not go on to develop enteric fever in the discovery cohort.

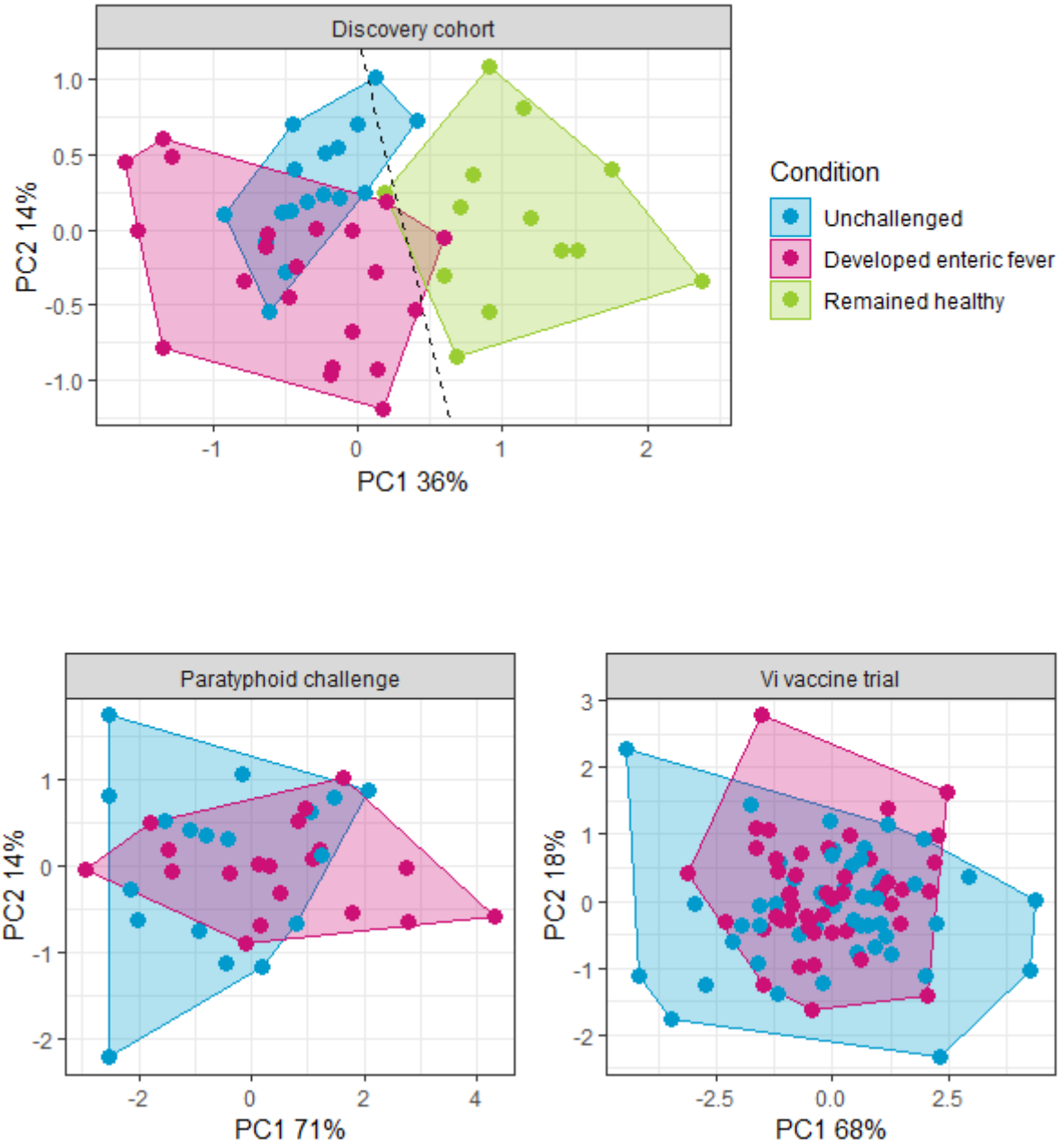
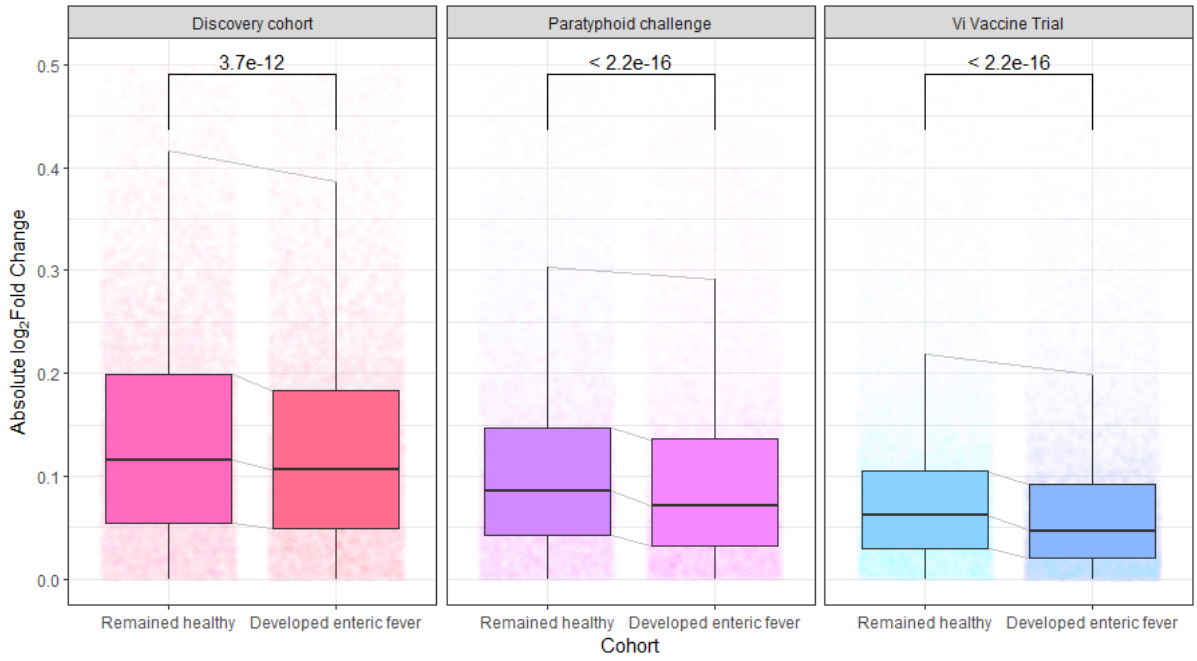


Figure 4.23: Distribution of absolute log_2fold changes in gene expression for those who did and did not go on to develop enteric fever for each cohort. A Mann–Whitney–Wilcoxon test was used to calculate the indicated p-values.



4.3.7 Vi-vaccinated individuals display less marked B cell module over-representation following challenge than recipients of a control vaccine

Gene set enrichment analysis was used to compare responses between the different vaccination groups in the Vi vaccine trial (Figure 4.24). In both Vi vaccine groups, gene sets relating to B cells were less over-represented than in the control group (FDR < 0.05). However, those who received the Vi-polysaccharide vaccine had greater over-representation of modules relating to TLR and inflammatory signalling (M16), monocytes (M11.0) and activated dendritic cells (M165) than the control group. Compared with the Vi-conjugate vaccine, the Vi-polysaccharide vaccine had greater enrichment of modules relating to IFN responses (M75, M11.1, M127) and antiviral responses (M150), while the conjugate arm showed more marked under-representation of modules relating to cell cycle (M4.1, M4.0, M46). A comparison was also carried out between those in the Vi vaccine control group (challenged with *S.* Typhi) and those challenged with *S.* Paratyphi A in the dose finding study (Figure 4.25). Those in the

paratyphoid challenge group had greater enrichment of gene sets relating to monocytes (M11, M118, S4, M64) and enrichment of suppression of MAPK signalling (M56).

Figure 4.24: Heat map showing the normalised enrichment scores of gene sets which were significantly over or under-represented (FDR < 0.05) in at least one of the Vi vaccine trial study arms 12 hours post-challenge relative to baseline. Over-represented gene sets are shown in red, and under-represented in blue.

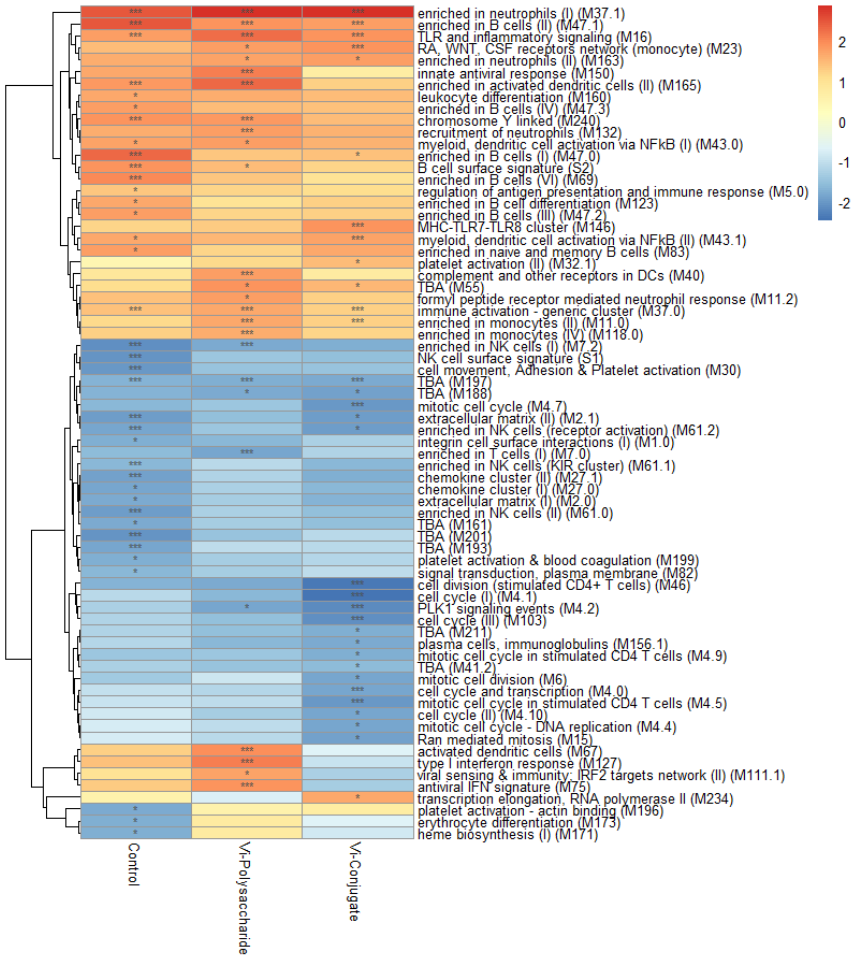
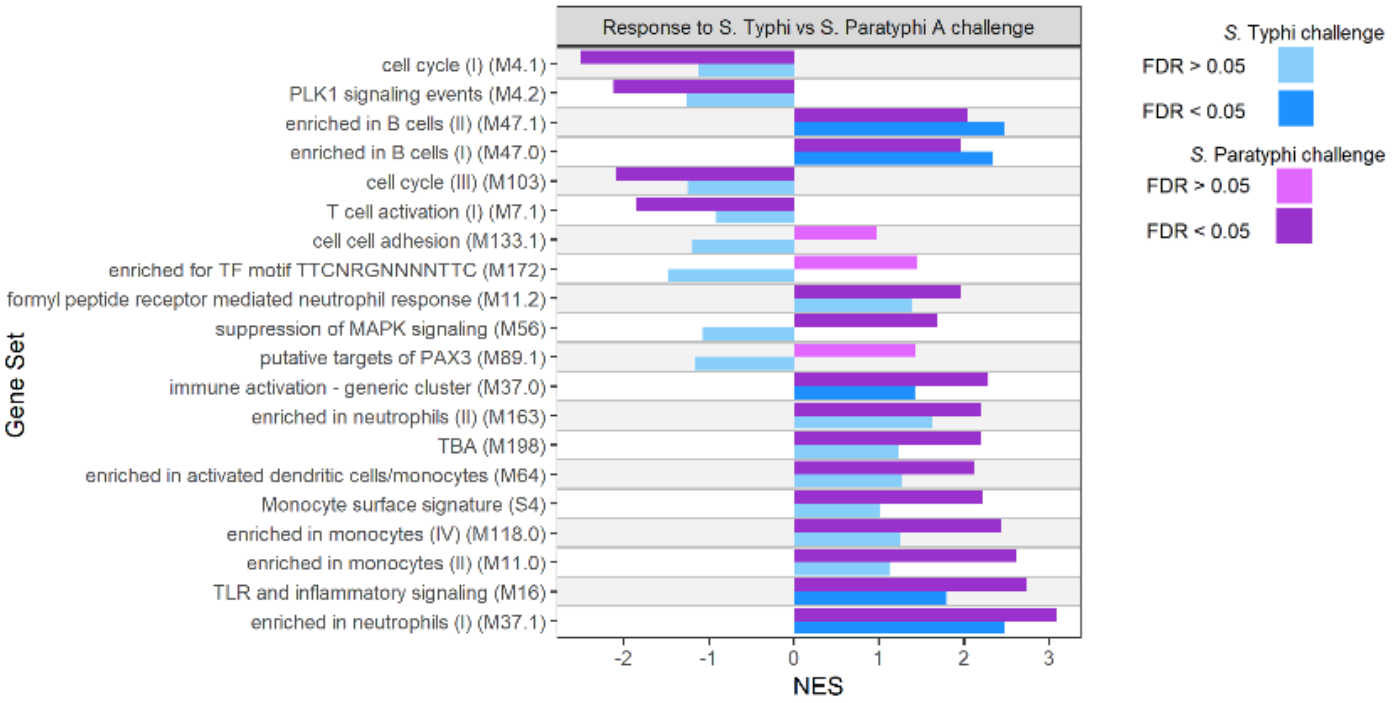


Figure 4.25: Normalised enrichment scores of gene sets significantly over- or under-represented in the 12 hours response to *S.* Typhi challenge relative to *S.* Paratyphi challenge (FDR < 0.01). Normalised enrichment scores for *S.* Typhi challenge are shown in blue and *S.* Paratyphi challenge in purple. Bars are filled in a lighter colour if they did not reach significance (FDR < 0.05) in each cohort.

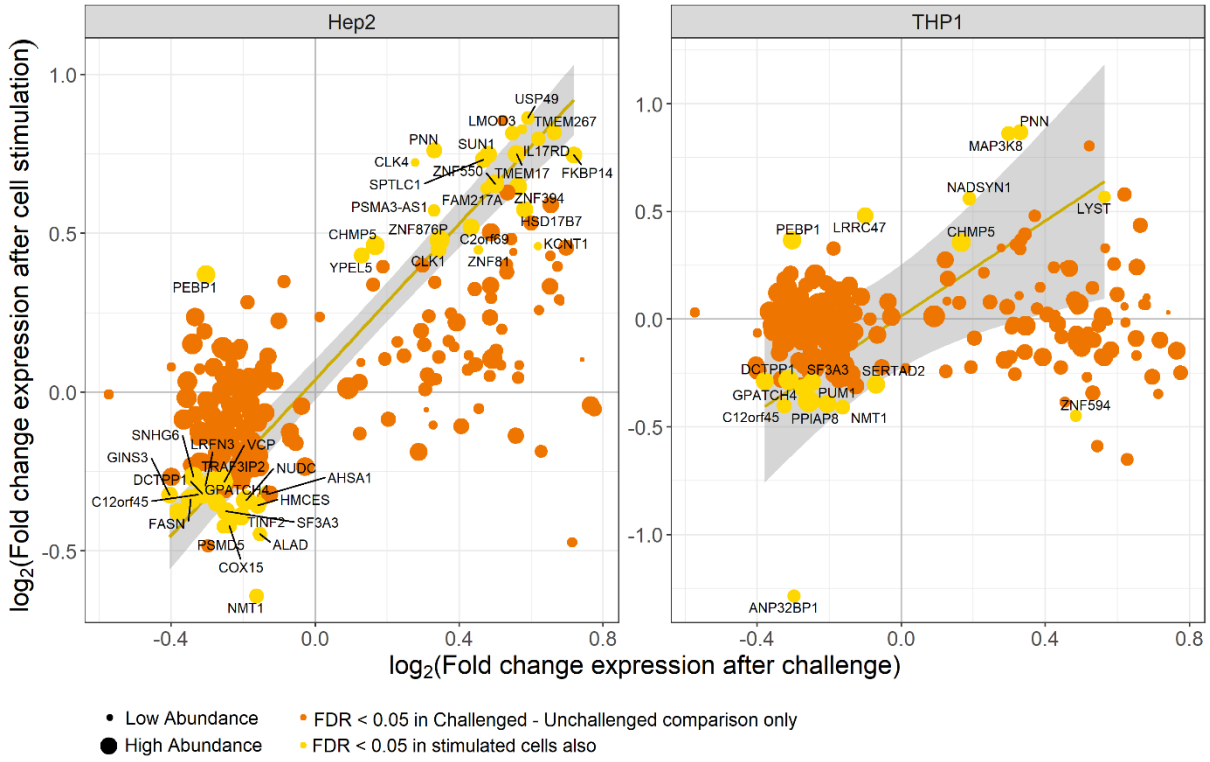


4.3.8 *In vitro* stimulation with *S.* Typhi and responses in blood in patients with inflammatory bowel disease give transcriptional changes correlating with that following human challenge

As many genes differentially expressed post-challenge may be attributed to changes in cell subset proportions in blood, changes in gene expression were compared with Hep2 and THP-1 cells stimulated *in vitro* with *S.* Typhi Ty2 derivative BRD948 (Figure 4.26) to identify genes which may be regulated at the transcriptional level. Amongst the 216 genes identified as challenge-specific, there was a significant correlation in $\log_2$(fold change) with both stimulated cell lines, although more markedly with Hep2 cells (ρ = 0.7 and $p = 1 \times 10^{-48}$) than THP-1 cells (ρ = 0.2, $p = 0.01$). As seen in blood from individuals challenged with *S.* Typhi, there was significant enrichment of HIF-1A and AhR binding sites amongst downregulated genes (Figure S4.10).

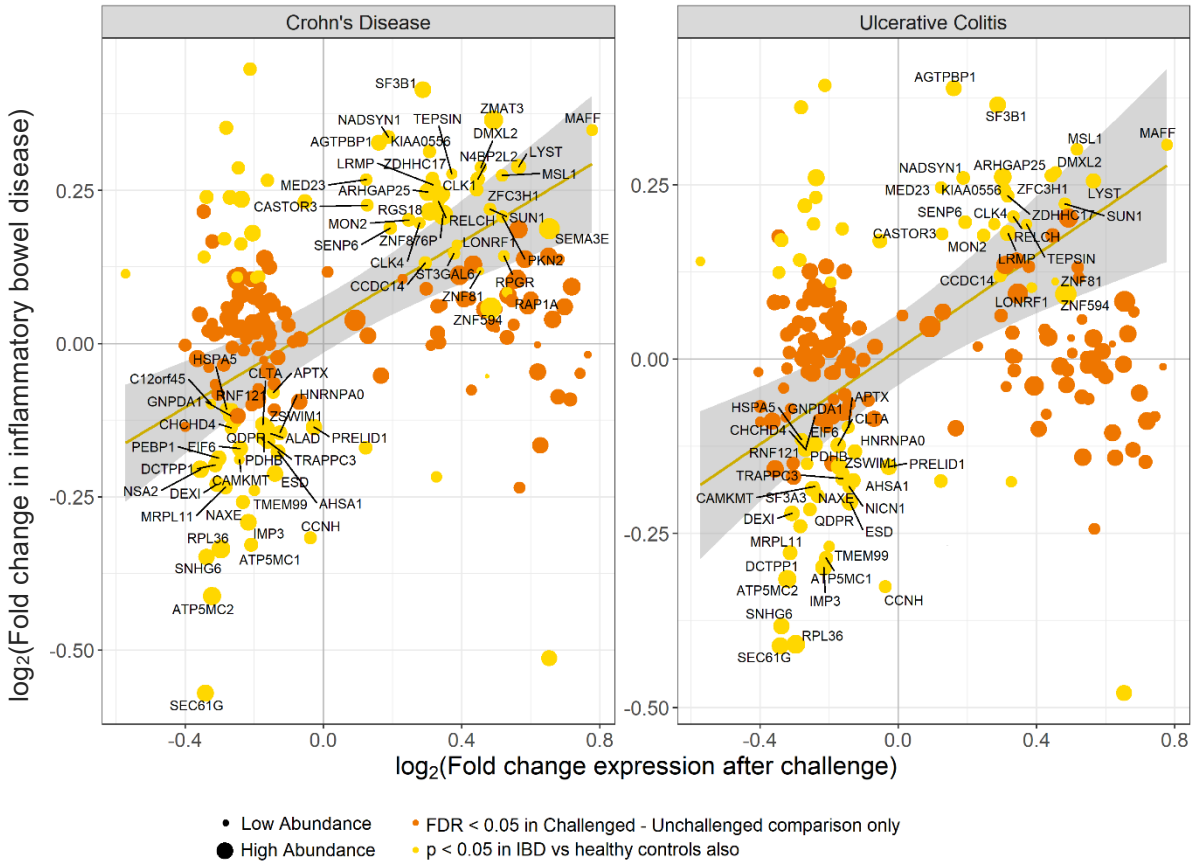
While in the Hep2 cells, expression of *AHR* itself did not change and *HIF1A* was upregulated (p = 0.01), in THP-1 cells HIF-1A did not change and AhR expression decreased (p = 0.06, Figure S4.11).

Figure 4.26: Correlation in log_2(fold change gene expression) between peripheral whole blood from volunteers challenged with Quailes strain *S.* Typhi and cell lines stimulated with *S.* Typhi Ty2 derivative BRD948 *in vitro*. Point size reflects abundance in the stimulated cell lines. Only genes which formed part of the challenge-specific response are shown, and those which were also differentially expressed by the stimulated cell lines are highlighted in yellow and labelled. A linear regression line fitted to genes differentially expressed both *in vivo* and *in vitro* is shown in orange with 95% shaded confidence intervals.



To then investigate whether the observed changes in gene expression may be reflective of intestinal inflammation, results were correlated with the log_2(fold change) in gene expression in the blood of patients with inflammatory bowel disease relative to healthy controls. For genes changed specifically in response to challenge, there was a significant relationship with changes seen in Crohn's disease (ρ = 0.3, p = 3 x 10^{-5}) and Ulcerative Colitis (ρ = 0.3, p = 0.01), both sharing upregulation of genes such as *MAFF* and *LYST* (Figure 4.27).

Figure 4.27: Correlation in log_2(fold change gene expression) between blood from volunteers challenged with *S.* Typhi 12 hours post-challenge relative to baseline, and peripheral whole blood from inflammatory bowel disease patients relative to healthy controls. Point size reflects abundance in the stimulated cell lines. Only genes which formed part of the challenge-specific response are shown, and those which were also differentially expressed in inflammatory bowel disease (unadjusted p value < 0.05) are highlighted in yellow and labelled. A linear regression line fitted to genes differentially expressed both early after typhoid challenge and in inflammatory bowel disease is shown in orange with 95% shaded confidence intervals.



4.3.9 Genes encoding early response cytokines CXCL1 and TGF-α are upregulated in response to human challenge

Of the early response plasma cytokines discussed in chapter 3, *CXCL1, TGFA* and *IL1RN* were consistently upregulated at the transcriptional level in whole blood 12 hours post-challenge (Figure 4.28). These cytokines were upregulated to a greater extent in participants who underwent challenge compared with unchallenged controls (unadjusted p = 0.002, 0.08 and

0.12 for *TGFA, CXCL1* and *IL1RN* respectively in the discovery cohort, linear modelling). However, the correlation between changes in whole blood gene expression and changes in plasma cytokine profile was poor (Figure 4.29).

Figure 4.28: Log_2(fold change in gene expression) for genes encoding "early response cytokines" 12 hours post-challenge relative to baseline. Changes where unadjusted p value < 0.05 are indicated with an asterisk.

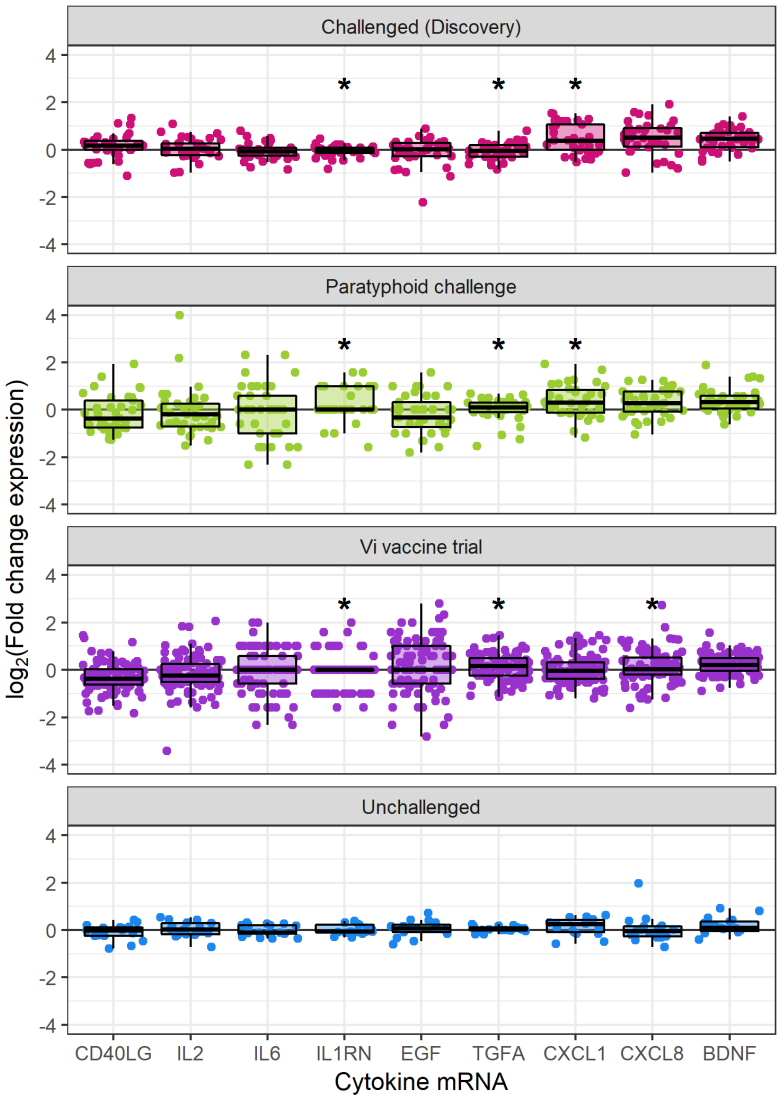
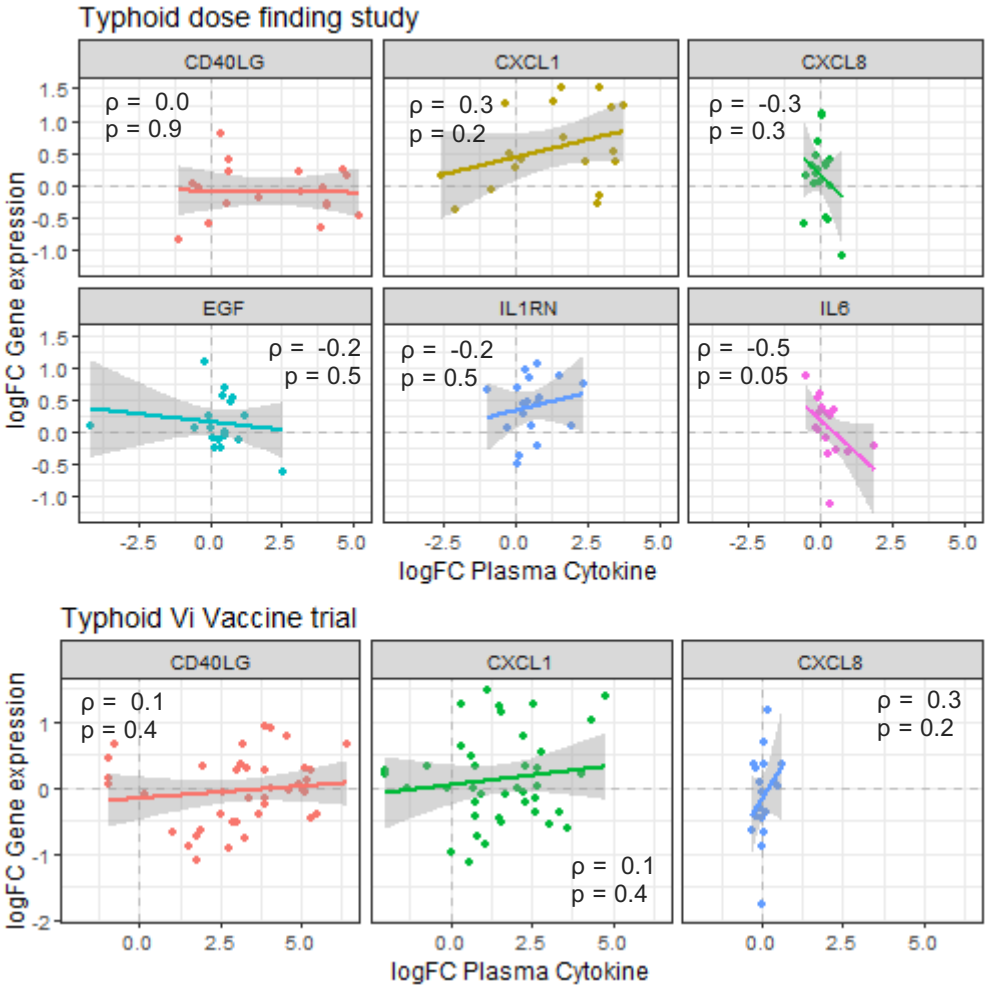


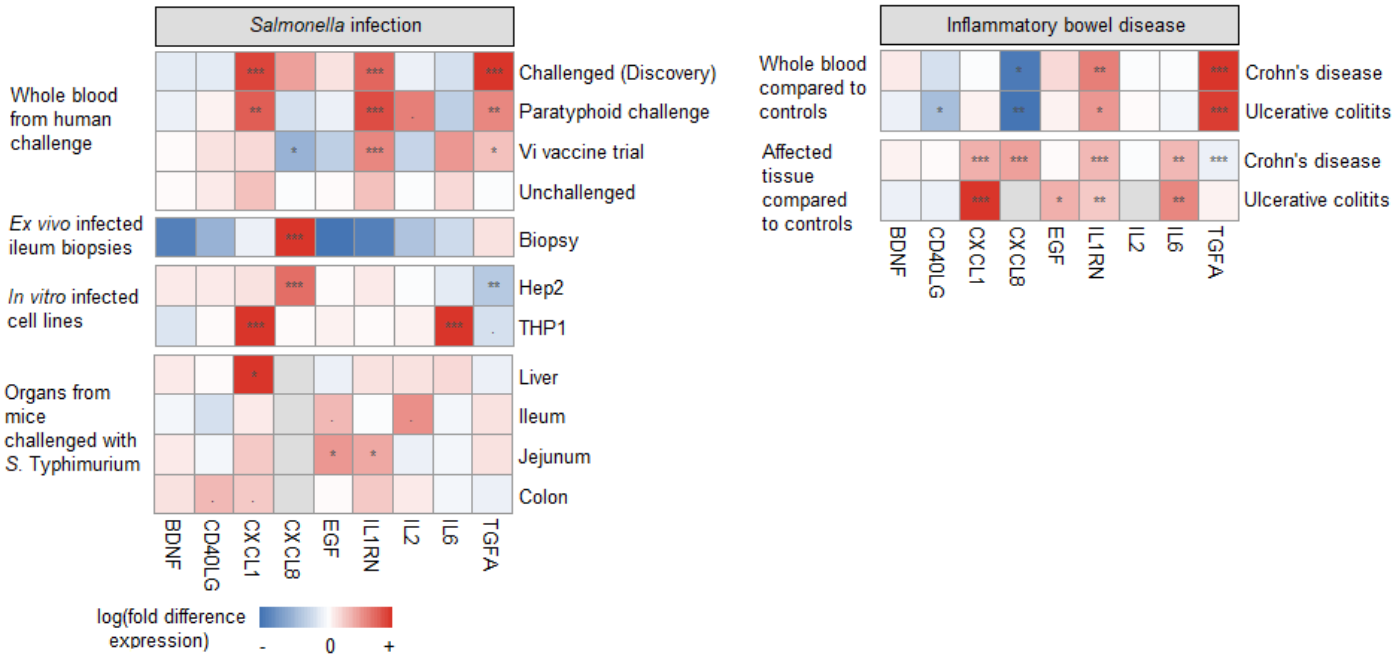
Figure 4.29: Correlation between the $\log_2$(fold change) in plasma cytokine levels and whole blood gene expression for early response cytokines where paired data was available. A linear regression line with 95% shaded confidence intervals is shown for each cytokine. The Pearson correlation coefficient (ρ) and p-value for a test of association between the two variables is indicated.



To investigate whether the intestinal mucosa and liver may also act as a source of these cytokines, five further publicly available gene expression datasets were used: data from human terminal ileum biopsies infected with 10^8 *S.* Typhi Ty2 strain for 2 hours; from the liver, jejunum, ileum and colon of mice infected with *S.* Typhimurium; from the whole blood of patients with Crohn's disease and ulcerative colitis; from the ileum of patients with Crohn's disease; and from the colon of patients with ulcerative colitis (Del Bel Belluz et al. 2016; Nickerson et al. 2018) (Figure 4.30). In addition to being upregulated in the whole blood of human challenge participants, *CXCL1* was produced by the liver of mice in response to *S.* Typhimurium infection, and in the intestinal mucosa of patients with inflammatory bowel disease. Despite a rise in

plasma EGF 12 hours post-challenge, *EGF* was not upregulated in whole blood, but was upregulated in the jejunum of infected mice and colon of ulcerative colitis patients, suggesting it may be intestinal in origin. While IL8 is not raised in the plasma early after challenge, *CXCL8* is upregulated in human intestinal biopsies following infection, as well as in the ileum of patients with Crohn's disease. In addition to being upregulated in whole blood for human challenge participants, *IL1RN* was upregulated in whole blood from IBD patients, the intestinal mucosa of IBD patients, and the jejunum of *S.* Typhimurium infected mice, suggesting it may originate from both peripheral blood and the intestine. *TGFA* was not only upregulated in whole blood 12 hours after human typhoid challenge, but also in the whole blood of IBD sufferers.

Figure 4.30: Up- and down- regulation of genes encoding "early response cytokines" in whole blood from human challenge participants 12 hours post-challenge, human ileum biopsies infected *ex vivo* with *S.* Typhi Ty2 strain for two hours, cell lines infected *in vitro* for one hour with *S.* Typhi Ty2 derivative BRD948, mice challenged with *S.* Typhimurium strain MC71-TT, whole blood from patients with inflammatory bowel disease, the ileum from patients with Crohn's disease and the colon from patients with Ulcerative Colitis. Colours are scaled for each dataset, with red indicating upregulation relative to baseline or uninfected controls, and blue downregulation. Significance of up- and down- regulation is indicated by the asterisks ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***).



4.5 Discussion

4.5.1 Changes in gene expression in the first 24 hours of challenge

During the period of infection where *S.* Typhi is thought to disseminate from the intestine via the peripheral blood we identified marked transcriptional perturbation. Due to practicalities of the study participants were always challenged in the morning, raising the issue of transcriptomic changes due to circadian rhythms. To address this issue, a publicly available dataset derived from a human circadian rhythm study was integrated with our discovery cohorts. In these unchallenged participants, only 16 genes were differentially expressed. While one approach would be to exclude these genes from further analysis in the challenged cohort, as the sample size of the control group was smaller than the challenged group, this would likely result in retaining circadian genes just below the threshold for significance. Therefore, we carried out a "difference of differences" comparison to identify genes specifically differentially expressed in response to challenge, sacrificing statistical power but reducing the number of false positives. As a result, any genes considered "challenge specific" tended to be those which changed in the opposite direction to that expected from circadian rhythms alone: we did not have enough power to identify genes which changed in the same direction but to a greater extent in the challenged cohort. In future, these limitations could be overcome by taking a blood sample from each participant the evening before challenge, thus allowing each participant's response to be normalised to their own circadian rhythm in a paired manner, or by randomising participants to receive challenge in the morning or evening.

Several of the most differentially regulated genes encode proteins with reported immunological functions, and therefore may be attractive targets of further research. Amongst the upregulated genes, *ST3GAL6* encodes a sialyl-transferase involved in the formation of sialyl-Lewis^{x} (CD15), playing a role in leukocyte homing (Glavey et al. 2019). The transcription factor *MAFF* has been identified as upregulated in myometrial cells after stimulation with pro-inflammatory cytokines (Massrieh et al. 2006), and itself regulates expression of CXCL1 (Saliba

et al. 2019), one of the cytokines increased in the plasma 12 hours post-challenge (Blohmke et al. 2016). *PTGR1* encodes an enzyme which inactivates chemotactic factor leukotriene B4, limiting inflammation and restricting mycobacterial growth (Sansonetti 2004). Amongst the downregulated genes are *TRAF3IP2*, an NF-κB activator, *PUM1*, a suppressor of the innate immune response (Liu et al. 2017), and *ZSWIM1*, a naïve CD4 T cell biomarker (Ko, Powell, and Hogarth 2014).

24 hours post-challenge the perturbation in the blood transcriptome appears to be subsiding. One potential explanation is that by this time point *S.* Typhi has already disseminated to the liver, spleen and bone marrow, as suggested by its resistance to chloramphenicol within 24 hours of challenge (Hornick et al. 1970). Here it is considered to be intracellular and therefore inaccessible to the immune system. The site of invasion is thought to be the small intestine, with intestinal perforations tending to occur in the terminal ileum (Chanh et al. 2004). Assuming the bacteria transit through the digestive tract at the same rate as ingested solids, we would therefore expect them to reach the site of infection within 4-12 hours (Y. T. Wang et al. 2015) and for the inoculum to have passed through the entire digestive tract approximately 30 hours post-challenge: thus it seems plausible for peak perturbation to be subsiding after a day. Furthermore, this result is consistent with plasma cytokines returning to baseline at 24 hours following a peak at 12 hours post-challenge (Blohmke et al. 2016). However, at 1-2 days post-challenge *S.* Typhi DNA can still be isolated from the blood (Darton et al. 2017), and positive stool cultures can persist throughout the incubation period (Hornick et al. 1970). However as neither method of detection is quantitative it is unknown at what point the number of bacteria in the blood or the stool peak, although the probability of shedding is highest in the first day after infection (Gibani, Voysey, et al. 2019). Another explanation for this observation is that circadian rhythms have not been entirely accounted for, or that co-normalisation and batch correction has not fully rectified the 24 hour samples having been profiled in a separate batch to baseline samples.

4.5.2 Functional analysis of differentially expressed genes

Pathway analysis did not identify any shared over- or under-represented pathways common to both the discovery cohort and the two validation cohorts, possibly due to pathway analysis relying on implementation of arbitrary cut-offs. However, binding sites for hypoxia inducible factor-1 (HIF-1A) and aryl hydrocarbon receptor (AHR) were consistently over-represented amongst genes downregulated in response to challenge, and this result was replicated *in vitro*. Although best known for its role in responding to hypoxia, HIF-1 regulates the bactericidal activity of phagocytes even in normoxia (Peyssonnaux et al. 2005), as well as chemoattractant secretion and phagocyte survival (Bhandari and Nizet 2014). AHR is involved in both endotoxin tolerance and resistance to bacterial infection (Quintana 2018). Amongst their targets specifically downregulated in response of challenge, pathways relating to antigen presentation and protein/amino acid metabolism were over-represented. At the transcriptional level *HIF1A* and *AHR* did not consistently rise or fall after infection, but could be undergoing post-translational regulation. TGF-β1, TNF-α and IL1β for example modulate the stability of HIF-1A protein without changes in mRNA expression (Bhandari and Nizet 2014). AHR on the other hand is directly activated by tryptophan catabolites such as kynurenine, a metabolite previously identified as being raised in the plasma during acute typhoid disease and which is correlated with disease severity (Blohmke et al. 2016).

To overcome the limitations of pathway analysis, gene set enrichment analysis, which does not rely on arbitrary thresholds, was carried out. Gene sets relating to neutrophils, monocytes and Toll-like receptor signalling were consistently over-represented, and those relating to T cells under-represented. Deconvolution by CIBERSORT attributed over-representation of neutrophil related modules to an increase in neutrophil fraction 12 hours post-challenge. Supporting this finding, estimated cell fractions correlated well with peripheral blood counts where available, and appeared to be stable, even in the presence of batch effects.

4.5.3 Interactions between *S.* Typhi and neutrophils

Given that *S.* Typhi does not induce intestinal neutrophil infiltration, is thought to disseminate systemically via Vi capsule-mediated evasion of neutrophils (Raffatellu et al. 2006), and results in neutropenia during acute disease (Abdool Gaffar et al. 1992), the estimated rise in neutrophils 12 hours post-challenge was initially surprising. We were therefore interested in whether neutrophils might phagocytose *S.* Typhi early after infection, and whether they are more likely to act as a vector for systemic infection in addition to mononuclear phagocytes, or rapidly kill invading bacteria.

Firstly, we established that uptake of bacteria was similar between granulocytes and monocytes, but low amongst lymphocytes. However as it is difficult to assess what constitutes "high" granulocyte uptake without reference to serovars which are known to cause intestinal neutrophil infiltration, we infected cells with different *Salmonella* strains and serovars, and found that in contrast to previous studies (Balakrishnan et al. 2016; Karlinsey et al. 2019), expression of the Vi capsule did not decrease uptake. However, this assay had several limitations. Firstly, there were differences in the intensity of bacterial staining between different strains and conditions. While this has been partially accounted for by normalising to the intensity of free bacteria, this is likely a major confounder. Secondly, background fluorescence varied between different cell populations. Finally, the growth rate of bacterial strains in the inoculum and in cell culture may have varied. These limitations may be addressed by switching to a microscopy based assay, in which distinct bacteria can be identified within cells; by optimising staining for each strain so as to be the same intensity; by expression of GFP by the bacteria or use of conjugated anti-LPS antibodies to increase staining specificity; and by cell lysis and agar plating to check for differences in bacterial growth rates in culture.

We then went on to investigate whether *S.* Typhi was able to evade neutrophil activation and killing, which would support the hypothesis that neutrophils can act as a *Salmonella* "safe-site".

In vitro, *S.* Typhi stimulation of white blood cells resulted in marked activation of neutrophils, resulting in CD62L shedding, upregulation of CD11b and CD11c, and an oxidative burst. The Vi capsule did appear to suppress the production of ROS by granulocytes. It has been previously been found that *S.* Typhi Quailes strain induced lower oxygen consumption in granulocytes than the less virulent 0-901 strain, a derivative of Ty2 lacking the Vi antigen (Kossack et al. 1981; Miller et al. 1972). Furthermore, in the presence of serum, an *S.* Typhi Δ*tviB-vexE* mutant elicited higher ROS production in HL-60 cells and primary neutrophils than the Vi-expressing parent strain (Hiyoshi et al. 2018). However, this represents the first time ROS production has been characterised simultaneously in three different cell populations, for a wide variety of strains in the absence of antibody. Four hours after *in vitro* infection the amount of *S.* Typhi viable within granulocytes was no different to the number surviving within monocytes. Although this may be attributable to some extent to an impure granulocyte fraction, as there are approximately four-fold as many granulocytes in this fraction this was unexpected. It may be that these represent a population who have survived long enough to upregulate certain virulence factors necessary to evade killing.

Culture of white blood cells in supernatants from infected cells also resulted in neutrophil activation, suggesting that soluble factors such as shed LPS or secreted cytokines play a role in the change of phenotype. This is consistent with literature showing that cell-free supernatants from an *S.* Typhi infected model of the intestinal mucosa stimulates granulocyte chemotaxis, with depletion of IL1β, IL6, IL8, TNF-α and CCL3 reducing chemotaxis (Salerno-goncalves et al. 2019). This may explain why a rise in estimated neutrophil fraction is seen in the blood despite bacteria only being present at a very low concentration during bacteraemia. However it was difficult to validate whether neutrophils were activated in human challenge participants *ex vivo*, as neutrophils are short-lived and prone to activation by uncontrolled factors.

4.5.4 Early differences between subgroups

At the individual gene and gene set level, no replicable differences were seen between those who went on to develop enteric fever and those who remained healthy. However, those who remained healthy tended to have a slightly greater magnitude of response, raising the possibility that a robust early response could be protective. Analyses on further cohorts will be necessary to confirm or refute this; however this is consistent with the previous finding that in those who remained healthy, there is a peak in the ability of monocytes and dendritic cells to bind to *S.* Typhi one day post-challenge (Toapanta et al. 2015).

There were also no differences at the individual gene level between *S.* Typhi and *S.* Paratyphi A challenge. This is in contrast to acute disease, where typhoid and paratyphoid give distinct metabolic profiles (Näsström et al. 2014), and *in vitro* infection, where *S.* Typhi and *S.* Paratyphi A induce distinct transcriptional responses (Salerno-goncalves et al. 2019). Those in the paratyphoid challenge group had greater enrichment of "suppression of MAPK signalling" (M56). *S.* Typhi and *S.* Paratyphi A both suppress expression of *MAP2K4, MAPK1, MAPK14, MAPK8* and *MAPK3* in an *in vitro* model of the intestinal mucosa, with suppression of *MAPK1, MAPK14, MAPK8* and *MAPK3* being greater for *S.* Paratyphi A (Salerno-goncalves et al. 2019). Thus this may represent an early immune evasion strategy by *S.* Paratyphi A. *S.* Paratyphi A challenge also gave greater enrichment of four monocyte modules. This could be attributed to the absence of a Vi capsule in *S.* Paratyphi A, leaving it more vulnerable to recognition by monocytes.

Surprisingly, gene set enrichment analysis suggested the control group had greater over-representation of B cell modules compared with the Vi-vaccinated groups. In the Vi-vaccine trial only one subset of participants exhibited a rise in Vi-specific IgG secreting cells seven days post-challenge: recipients of the conjugate vaccine who remained healthy (unpublished data). Even in this group, a secondary B cell response would be unlikely to be detectable in the first 12 hours after challenge (Blanchard-rohner et al. 2009). However, the Vi polysaccharide vaccine group did show greater enrichment 12 hours post-challenge of two modules relating to

monocytes and activated dendritic cells, which may be reflective of the increase in antibodies with the capacity to induce phagocytosis observed after vaccination (unpublished data).

4.5.5 Transcriptional upregulation of genes in response to *S.* Typhi infection

Challenge responses were compared with Hep2 and THP-1 cells stimulated *in vitro* to identify genes likely differentially regulated at the transcriptional level, rather than occurring as a result of changes in cell populations. Although stimulated with an attenuated *S.* Typhi strain, there was still a significant correlation between changes in gene expression. Surprisingly, the correlation was much stronger with the epithelial Hep2 cell line than the monocyte-like THP-1 cell line. Of particular interest was the increase in *LYST* in the THP-1 cells. Mutations in *LYST* give rise to Chédiak–Higashi syndrome, a disease characterised by a decrease in phagocytosis and susceptibility to bacterial infections (Lozano et al. 2014). Furthermore, *LYST* knock-out mice orally challenged with *S.* Typhimurium suffer from higher bacterial loads in the liver and spleen (Westphal et al. 2017). Although its function has not yet been fully elucidated, LYST is able to limit *Leishmania* infection by restricting the size of parasite-containing intracellular vacuoles (J. Wilson et al. 2008), and also regulates TLR3 and TLR4-mediated inflammatory responses.

4.5.6 Correlation with changes in the blood transcriptome in inflammatory bowel disease

To investigate whether changes in the blood transcriptome may reflect intestinal inflammation, the $\log_2$(fold change) of challenge-specific genes 12 hours after baseline was compared with differences in the blood transcriptome of inflammatory bowel disease patients relative to healthy controls. A significant correlation was observed, suggesting that host-pathogen interactions in the intestine could feasibly affect gene expression in the blood. This correlation was more significant for Crohn's disease than Ulcerative colitis, which may reflect that whereas Ulcerative colitis affects the bowel, the terminal ileum is both the most commonly affected area

in Crohn's disease and the putative invasion site of *S.* Typhi (Goulart et al. 2016). The mechanism by which IBD affects extra-intestinal sites is not well understood, but may occur due to migration of lymphocytes activated in the gut to different tissues (Eksteen et al. 2004).

4.5.7 Expression of "early response" cytokines at a transcriptional level

Differential regulation of genes encoding "early response" cytokines, discussed in Chapter 3, were also examined and correlated poorly with changes in plasma levels 12 hours post-challenge. This is consistent with previous literature suggesting that in humans, the correlation between mRNA and protein expression is generally below an R^2 value of 0.4 (Abreu et al. 2009). This may be due to a number of factors. Firstly, there is likely a lag time of several hours between transcription and translation, and once expressed proteins are more stable (Cheng et al. 2016; Hoogendijk et al. 2019). Secondly, protein levels may be post-transcriptionally regulated by microRNAs, RNA binding proteins, translation rate and degradation rate (Liu, Beyer, and Aebersold 2016). Finally, the correlation between intracellular and secreted cytokines is low, suggesting that secretion into the plasma is regulated (Song et al. 2019), and cytokines secreted by non-peripheral blood cells may be present in the blood.

We then used publicly available data to assess whether other tissues may be responsible for secretion of "early response" cytokines into the plasma. *S.* Typhi is thought to invade the intestinal mucosa, disseminate via the blood and then replicate in the liver, spleen and bone marrow (Dougan and Baker 2014). Of the early response cytokines, *TGFA, CXCL1* and *IL1RN* were upregulated in whole blood 12 hours post-challenge. In both chronic liver disease and inflammatory bowel disease serum levels of IFN-γ, IL1β and TNF-α are raised relative to healthy controls, suggesting that inflammation at these sites can affect cytokine levels in the peripheral blood (Singh et al. 2016; Yamauchi et al. 1994). Of the "early response" cytokines, *TGFA* and *IL1RN* were upregulated in the peripheral blood of inflammatory bowel disease patients relative to healthy controls; thus, is it possible this change in expression post-challenge occurs as a

result of leukocytes trafficking from the intestine into the circulation. While not upregulated in the peripheral blood of IBD patients, *CXCL1* was upregulated by monocyte-like THP-1 cells in response to direct stimulation with *S.* Typhi.

Several plasma cytokines raised 12 hours post-challenge such as *EGF* and *IL6* were not upregulated at the transcriptional level peripheral blood, and therefore might occur due to secretion of cytokines into the circulation by the liver, spleen, intestine or bone marrow. This may also partially explain why correlation between the gene expression and plasma protein levels of each cytokine was poor. *CXCL1*, *EGF, IL1RN* and *IL6* were upregulated in the intestinal mucosa of inflammatory bowel disease patients, and *EGF* and *IL1RN* in the jejunum of mice. Furthermore, *CXCL1* was upregulated in the liver of challenged mice. While publicly available data are useful here for hypothesising where early response cytokines may originate from, neither *ex vivo* infection of intestinal biopsies nor *in vivo* infection of mice are perfect models of human enteric infection. Mice were infected with *S.* Typhimurium for 60 days, and thus changes in gene expression represented a response to chronic rather than early infection. Furthermore, both studies used different strains to human challenge agents *S.* Typhi Quailes strain and *S.* Paratyphi A strain NVGH308. Thus further work is necessary to ascertain the contribution of the intestine and liver to changes in cytokine profile.

4.5.8 Conclusion

Overall, there appears to be marked and consistent transcriptional perturbation in response to *S.* Typhi and *S.* Paratyphi A challenge, accompanied by downregulation of HIF and AHR targets and a rise in neutrophil fraction. This is the first evidence of an early response to *S.* Typhi infection at the transcriptional and cellular level. Although neutrophils have a greater killing capacity for *S.* Typhi than monocytes, we found that there were still bacteria surviving within the granulocyte fraction 4 hours post-infection *in vitro*, and that while the Vi capsule did not inhibit uptake, it did reduce production of ROS, raising the question of whether *S.* Typhi is able

to evade neutrophil killing and utilise these cells as an additional vector for dissemination. At this early point in infection we were not able to predict outcome from transcriptional changes, although there is a trend towards larger responses in those who remain healthy, suggesting that a robust early response may be protective.

Chapter 5 | Genetic susceptibility to enteric fever

5.1 Introduction

While many behavioural and environmental risk factors have been identified for enteric fever, such as lack of handwashing, poor sanitation and flooding, these factors cannot explain why in a controlled human infection model one participant challenged with *S.* Typhi develops typhoid fever while another remains healthy (Saha et al. 2018). The outcome of exposure likely depends on the interplay between environment and host genetics. Since 2007, genome-wide association studies (GWAS) have been used to find associations between genetic variants and complex non-Mendelian traits, with the aim of identifying genes which give insight into the pathology of a disease (Visscher et al. 2017). For example, before 2007 the only variant known to be associated with Ankylosing spondylitis (AS) was HLA-B*27 (Visscher et al. 2012). However GWAS led to the discovery of a protective *IL23R* allele which impairs IL-23-induced Th17 responses, suggesting the IL-23/IL-17 axis contributes to disease. It has since been found that HLA-B*27 misfolding triggers ER stress and enhances IL-23 production (Smith and Colbert 2014), and that treatment with an anti-IL-17A monoclonal antibody significantly abrogates symptoms (Sieper et al. 2017). However, in many cases GWAS highlights variants of unknown biological relevance, leading to an increase in the popularity of expression quantitative trait loci (eQTL) studies, where variants associated with gene expression are identified. Furthermore, effect sizes are generally small, and a certain locus will account for only a small proportion of the heritability of a polygenic disease.

Studies on host susceptibility to typhoid fever (outlined in table 5.1), both in the pre- and post-GWAS era, have primarily focused on the major histocompatibility complex (MHC) locus, also known as the human leukocyte antigen (HLA) locus. This highly polymorphic region of chromosome 6 is subdivided into three classes: HLA Class I, involved in endogenous antigen

presentation; HLA Class II, involved in exogenous antigen presentation, and HLA Class III, encoding TNF-α, TNF-β, several complement components, and other immune genes (Janeway et al. 2001). Targeted genotyping of polymorphisms in the Class II and III regions initially identified significantly different allele frequencies in HLA-DRB1, TNF-α and TNF-β between typhoid cases and controls in Vietnam (Dunstan, Stephens, et al. 2001). HLA-DRB1*12 was subsequently identified as being protective against typhoid fever complications such as gastrointestinal bleeding (Dharmana et al. 2002), and a TNF region haplotype as associated with both protection and a greater *ex vivo* TNF-α response to LPS (Dunstan et al. 2007). Finally, a GWAS identified the HLA-DRB1*04:05 allele as conferring resistance against typhoid (Dunstan et al. 2014). Although no MHC class I variants have previously been identified which affect enteric fever susceptibility, HLA-B*27, the same HLA type associated with Ankylosing spondylitis, is associated with non-typhoidal *Salmonella*-induced reactive arthritis (see table 5.1). *In vitro*, expression of HLA-B*27 appears to enhance the intracellular replication of *S.* Typhimurium, which may be a result of the unfolded protein response creating a hospitable environment for bacterial growth (Antoniou et al. 2019; Penttinen et al. 2004).

Outside the HLA locus, the role of several further genes in typhoid susceptibility have been investigated. Polymorphisms in natural resistance-associated macrophage protein 1, although associated with murine susceptibility to *S.* Typhimurium, did not appear to play a role in human resistance to typhoid fever (Dunstan, Ho, et al. 2001). A nonsense mutation in TLR5, investigated due to its association with legionnaires disease, also occurred at the same frequency between cases and controls (Dunstan et al. 2005). While one polymorphism in TLR4 was identified as being of greater frequency amongst cases ($p = 0.04$), this was not significant after adjusting for multiple testing (Nguyen et al. 2009). However a polymorphism in an intronic region of cystic fibrosis transmembrane conductance regulator, involved in intestinal uptake of *S.* Typhi, was successfully identified as associated with typhoid fever in Indonesia (van de Vosse et al. 2005).

Table 5.1: Literature associating human genetic variants with *Salmonella* resistance or susceptibility				
Clinical syndrome	Cohort	Method	Findings	Reference
Enteric fever	432 cases and 2011 controls from Vietnam, validated in 595 cases and 386 controls from Nepal, and 151 cases and 668 controls from Vietnam	Genotyping using Illumina OmniExpress BeadChip and Illumina 660W BeadChip, with HLA imputation using SNP2HLA	rs7765379 and HLA-DRB1*04:05 were associated with resistance to enteric fever	(Dunstan et al. 2014)
	103 participants from the Paratyphoid dose finding study and Vi vaccine trial	Illumina HiSeq 200 V4 and HLA imputation using HISAT-genotype	The following thesis chapter	
Typhoid fever only	112 cases and 77 controls from Dong Thap, Vietnam, and 105 cases and 211 controls from Dong Nai, Vietnam	PCR	No association between *NRAMP1* alleles and susceptibility	(Dunstan, Ho, et al. 2001)
	111 cases and 77 controls from Dong Thap, Vietnam, and 82 cases and 251 controls from Dong Nai, Vietnam	PCR	HLA-DRB1*0301/6/8, HLA-DQB1*0201-3, and TNFA*2 were associated with susceptibility, and HLA- DRB1*04, HLA-DQB1*0401/2, and TNFA*1 with resistance	(Dunstan, Stephens, et al. 2001)
	565 cases and 281 controls from Vietnam	PCR	TLR5 deficiency has no effect on typhoid fever susceptibility	(Dunstan et al. 2005)
	380 cases and 380 controls from Vietnam	PCR	Seven SNPs surrounding the *TNF* locus were significantly different between cases and controls, and those with a protective haplotype had a low *ex vivo* TNF-α response to LPS	(Dunstan et al. 2007)
	20 children with typhoid, 30 carriers, and 50 controls from Vietnam	PCR and restriction fragment length polymorphism analysis	Typhoid carriers have somewhat more *TLR4* gene polymorphism than those with symptomatic typhoid	(Nguyen et al. 2009)
	496 cases and 500 controls from Vietnam	PCR	rs8060947, a SNP associated with S. Typhi invasion and *VAC14* expression, was associated with susceptibility to typhoid fever	(Alvarez et al. 2017)
	96 participants from the typhoid dose	Genotyping using Illumina		

	finding study and typhoid oral vaccine trial	OmniExpress BeadChip and HLA imputation using SNP2HLA	The following thesis chapter	
Severe typhoid fever	44 patients with non-complicated and 19 with severe typhoid fever from Indonesia	PCR	HLA-DRB1*12 is associated with resistance to severe typhoid fever	(Dharmana et al. 2002)
	426 with mild typhoid fever and 35 with severe from Indonesia	PCR and restriction fragment length polymorphism analysis	Polymorphisms in NOD2, but not VDR or NRAMP1, were associated with severe typhoid fever	(Dwiyanti et al. 2017)
Non-typhoidal *Salmonella* bacteraemia	1885 cases and 3000 controls from Kenya (discovery), 532 cases and 1444 controls from Kenya (replication), 150 cases and 339 controls from Malawi (replication)	Affymetrix SNP 6.0 chip	rs13390936 in *STAT4* is associated with susceptibility to iNTS and reduced serum IFNγ levels in those infected	(Gilchrist et al. 2018)
Salmonella-induced reactive arthritis	63 patients with *Salmonella*-induced reactive arthritis from Finland	Micro-lympho-cytotoxicity HLA typing	88% were HLA-B27$^+$	(Leirisalo-Repo et al. 1997)
	45 patients with *S.* Bovismorbificans-induced gastroenteritis from Finland	Micro-lympho-cytotoxicity HLA typing	45% of patients with reactive arthritis and 0% of patients without were HLA-B27$^+$	(Mattila et al. 1998)
	198 patients with culture-positive acute *Salmonella* infection and 100 controls from Finland	PCR and flow cytometry	19.2% cases and 13% controls were HLA-B27$^+$ HLA-B27$^+$ patients more frequently reported *Salmonella*-induced joint symptoms than HLA-B27$^-$ patients (e.g. 29% vs 4.4% reported joint stiffness) and had more severe joint symptoms	(Ekman, Kirveskari, and Granfors 2000)
	46 patients with acute *S.* enteritidis-induced gastroenteritis and 91 controls from Canada	PCR	HLA-B27 was associated with susceptibility to *Salmonellosis* but not *Salmonella*-induced arthritis	(Rohekar et al. 2008)
	125 patients with *Salmonella*-positive stool culture from Finland	Not specified	42% of those with *Salmonella*-induced reactive arthritis were HLA-B27$^+$	(Tuompo et al. 2013)

More recently, transcriptomics has been used to investigate genetic susceptibility to infection, at times in conjunction with genotyping. In an Enterotoxigenic *Escherichia coli* challenge

model, 29 genes were differentially expressed at baseline between those who developed diarrhoea after exposure and those who did not (Yang et al. 2016). Expression quantitative trait loci (eQTL) analysis, in which polymorphisms that correlate with gene expression are identified, permits identification of genetic variants with a more defined function, and requires a smaller sample size than GWAS (Nica and Dermitzakis 2013). Through eQTL analysis on hundreds of genetically distinct lymphoblastoid cell lines, a single nucleotide polymorphism (SNP) associated with expression of *VAC14*, a regulator of cholesterol metabolism, and *S.* Typhi invasion was identified (Alvarez et al. 2017). Examining the effect of this SNP (rs8060947) in the Vietnam case-control dataset, it was confirmed to be associated with typhoid susceptibility in a human population (Alvarez et al. 2017).

The typhoid human challenge model has enabled several characteristics existing prior to exposure to be identified as associated with outcome of challenge. Those with a *Methanobrevibacter* dominated faecal transcriptome for example appear to be (non-significantly) more resistant to infection (Zhang et al. 2018). Baseline levels of multifunctional $CD8^+$ cells have also been previously related to outcome, although whereas one study suggested these cells were protective, a subsequent study suggested an association with susceptibility (Fresnay et al. 2016, 2017). While these factors are non-genetic, *Methanobrevibacter* in the faecal microbiota is estimated to be 21% heritable (Lim et al. 2017), and CD8+ T cell numbers 56% heritable (Hall et al. 2000).

In the following chapter we explore genetic susceptibility to enteric fever using the human challenge model. While in field studies genetic heterogeneity in the pathogen is a major confounder, for example in tuberculosis where host SNPs have been identified which only predispose individuals to a particular bacterial strain, here the *S.* Typhi Quailes strain is consistently used as a challenge agent. Furthermore, in the human challenge model all participants are exposed to the pathogen, whereas in the field the “controls” may have been previously infected, or may have avoided infection due to lack of environmental exposure

rather than innate resistance (Mozzi, Pontremoli, and Sironi 2018). Here we exploit this unique setting to investigate how differences in host baseline transcriptome, HLA type and genome relate to outcome of challenge, as well as the relationship between genetic variants and post-challenge gene expression.

5.1.1 Research questions

In the following chapter we attempt to address the following research questions with regards to genetic susceptibility to enteric fever:

Question: Is gene expression at baseline related to outcome of challenge?
Approach: We use several differential gene expression analysis tools to screen for differences in the baseline transcriptome within each challenge cohort, as has been previously performed for enterotoxigenic *E.* coli (Yang et al. 2016). As mRNA often does not correlate well with protein, we then perform an ELISA to investigate whether the most biologically relevant finding is replicated at the protein level.

Question: Given the unique advantages of the human challenge model, is it possible to identify biologically relevant loci associated with susceptibility to infection using a smaller sample size than typical GWAS studies?
Approach: Genotyping was performed on two challenge cohorts, allowing us to investigate SNPs throughout the genome for novel associations with outcome of challenge. HLA imputation was carried out on four challenge cohorts using genotype and RNA-sequencing data, increasing the sample size and reducing the burden of multiple testing relative to genome wide association. We perform a logistic regression in order to identify SNPs and HLA types associated with outcome of challenge, allowing covariates such as vaccination status to be included in the model. We then validate one of the most biologically relevant findings using a knock out cell line. Finally, we investigate whether any candidate HLA types are related to

mRNA expression of their respective gene, which may affect antigen presentation, or susceptibility at a cellular level to *S.* Typhi invasion.

Question: Can we replicate the finding that HLA-DRB1*12 is associated with enteric fever severity, and identify novel associations with changes in body temperature during acute disease?

Approach: As discussed in Chapter 3, in the absence of complicated typhoid cases in the human challenge model we use peak temperature as a proxy for severity, and carry out a linear regression to identify associations between HLA type and this continuous variable.

Question: Does a participant's genetics affect how they respond to challenge?
Approach: We carry out an eQTL analysis to identify associations between certain SNPs and response to challenge, both 12 hours post-infection and during acute disease, in order to investigate whether host genetics play a role in variability in response to infection.

5.2 Materials and methods

5.2.1 Association between baseline blood transcriptome and outcome

Sample processing, alignment and data processing of gene expression data took place as described in Chapter 3. In the cohorts profiled by microarray (the typhoid dose finding study and typhoid oral vaccine trial), association between baseline blood transcriptome and outcome was carried out using the limma package, incorporating dose as a covariate into the linear model. For the cohorts profiled by RNA-sequencing (the paratyphoid dose finding study and Vi vaccine trial), association testing was carried out in DeSEQ2, edgeR and limma, including dose, sequence pool and vaccination status as covariates where appropriate. P values were adjusted to account for multiple testing using a Benjamin-Hochberg correction.

5.2.2 Plasma C1q quantification

Blood samples were collected in heparin tubes by OVG staff from participants in the paratyphoid dose finding study. Samples were centrifuged for 10 minutes at 3,000rpm. Protease inhibitors were then added, and the plasma aliquoted then stored at -80°C. C1q levels were quantified using a Complement C1q ELISA Kit (ab170246, Abcam). Briefly, plasma samples were diluted 1:100,000 in the provided diluent, and 50µl added to ready coated plates in duplicate. After 2 hours incubation at room temperature, the plate was washed and biotinylated anti-C1q antibody added. After a further hour at room temperature, the plate was washed and incubated with 50µl Streptavidin-Peroxidase Conjugate. 30 minutes later the plate was washed and substrate was added to the well. After 10 minutes stop solution was added and absorbance values measured at 450nm using a Biotek ELx808 reader. Duplicates were averaged, and a 5-parameter logistic regression fit to the standard curve to estimate concentration. A Mann–Whitney–Wilcoxon test was used to assess whether the difference between outcome groups was statistically significant.

5.2.3 Genotyping

Genotyping was carried out by the Wellcome Trust Centre for Human Genetics on DNA samples from the typhoid dose finding (n=26) and typhoid oral vaccine trial (n=70) using an Illumina OmniExpress-24 v1.0. 716,503 SNPs were profiled in total. With the HapMap CEU dataset as a reference, SNP2HLA software (Jia et al. 2013) was used to impute single nucleotide polymorphisms in the HLA region and identify HLA alleles. Data cleaning for association analysis was carried out in PLINK (Purcell et al. 2007). SNPs with missingness > 10% (2,738), minor allele frequency < 0.1 (196,798) and Hardy–Weinberg equilibrium < 1×10^{-3} (682) were excluded, leaving 516,984 SNPs. One sample was excluded due to high missingness (>10%) and five due to outlying principal components (>2 standard deviations from the mean in component 1 and 2) for association analysis. Sex as determined by X chromosome heterozygosity matched the sample information given. Association analysis was carried out using a logistic regression

model in PLINK, with challenge dose, vaccination status and sex as covariates. Plots were generated using qqman, ggplot2 and LocusZoom (Pruim et al. 2011).

5.2.4 HLA typing from RNA-sequencing

RNA-sequencing data from the paratyphoid dose-finding and Vi vaccine trials was obtained as described in Chapter 4. Paired fastq files were aligned to a pre-built graph reference (ftp://ftp.ccb.jhu.edu/pub/infphilo/hisat-genotype/data) using HISAT2, followed by extraction of HLA-aligning reads. HLA typing and assembly was then carried out using HISAT-genotype (Kim, Paggi, and Salzberg 2018).

5.2.5 Association between HLA type and outcome, severity and gene expression

HLA type data from all cohorts were combined. Analyses were initially limited to HLA types at a resolution of 1 digit and with a frequency >3 (>25% dosage) in our cohort. A logistic regression model was used to identify HLA types associated with outcome (1 = diagnosed with enteric fever, 0 = remained healthy). Linear regression was used to identify HLA types associated with maximum temperature in those who developed disease, and HLA types associated with gene expression. Vaccination status and challenge dose were included as covariates. Statistical tests were carried out in R.

Publicly available data on the HLA type, baseline gene expression (as measured by microarray) and susceptibility to *S.* Typhi infection of Hapmap human lymphoblastoid cell lines was obtained from the dbMHC database final archive, gene expression omnibus (GEO accession: GSE6536) and supplementary data from a publication investigating *in vitro* susceptibility to *S.* Typhi infection (Alvarez et al. 2017). For the gene expression data, samples normalised across the population were retained, re-normalised by quantile normalisation, then filtered to intensity values corresponding to HLA-A, HLA-B, HLA-C and HLA-DRB1.

5.2.6 Intracellular survival of *S.* Typhi in HLA-B*27^{+} cells

HLA-B*27:05^{+} HeLa and C1R cells generated using lentiviral constructs were provided by the Bowness Group (Chen et al. 2017). Parent and HLA-B*27:05^{+} cells were seeded in a 96 well plate at a density of 100,000 cells per well. A frozen glycerol stock of 5 x 10^{8} CFU/ml *S.* Typhi Quailes strain was thawed and washed twice with RPMI. Cells were inoculated with at an MOI of 10 or 50 in triplicate. After one hour, gentamycin was added at a concentration of 200ug/ml. 24 hours post-inoculation cells were washed twice with RPMI then resuspended in 50ul 1% Triton-X100. After two minutes, lysates were serially diluted in PBS and plated onto tryptone soya agar. Colonies were counted following overnight incubation at 37^{o}C. A t-test was used to assess the difference in colonies between parent and HLA-B*27:05^{+} cells.

5.2.7 Expression quantitative trait loci analysis

Genotyping and transcriptomic data from the typhoid dose finding and typhoid oral vaccine trial were processed as outlined in 5.2.1 and in Chapter 4 respectively. In addition to the baseline and 12 hour post-challenge data analysed in Chapter 4, transcriptional data from acute disease was also quantile normalised, batch corrected, collapsed to genes, reannotated, and filtered (genes expression significantly ($p < 0.05$) above control probes in >25 samples). The R package MatrixEQTL (Shabalin 2012) was used to find linear associations between genetic variants and normalised gene expression at baseline, 12 hours post-challenge, and acute disease, as well as changes in gene expression at these time points relative to baseline ("response eQTLs"). Cohort, challenge dose, vaccination status and sex were specified as covariates in the model. As proximal promoter regions tend to occur within 1kb upstream of a gene, "cis"-eQTLs were defined as being within 1kb of a gene as annotated using Ensembl (genome assembly GRCh37), and trans-eQTLs as falling outside of this region (Pande et al. 2018). P values were adjusted to account for multiple testing using a Benjamin-Hochberg correction.

5.3 Results

5.3.1 Higher baseline expression of complement-related genes *C4BPA* and *C1QC* are associated with protection following paratyphoid challenge

After adjusting for multiple testing, in the cohorts profiled by microarray (typhoid dose finding study, typhoid oral vaccine trial) no genes were differentially expressed at baseline between those diagnosed with enteric fever and those who remained healthy as determined using limma (Figure S5.1). For the two cohorts profiled by RNA-sequencing (paratyphoid dose finding study and Vi vaccine trial), several different methods of assessing significant differential expression were compared: DeSEQ2 (Wald test), edgeR (likelihood ratio test and quasi-likelihood F-tests) and Limma-VOOM (t-test). DeSEQ2 and both edgeR tests indicated higher expression levels of *C4BPA, SLC37A2* and *C1QC* in those who remained healthy following paratyphoid challenge (FDR < 0.05, Table 5.2, Figure S5.2). These methods also suggest that in those who were given the Vi-polysaccharide vaccine, those who succumbed to *S.* Typhi infection expressed higher levels of *GLDC*. In the same subgroup, higher levels of *ISG15* and *IFI27* were associated with protection using the edgeR likelihood ratio test. Limma-VOOM gave different results, suggesting that *ZNF208* was more highly expressed in those protected from typhoid fever in the Vi vaccine study.

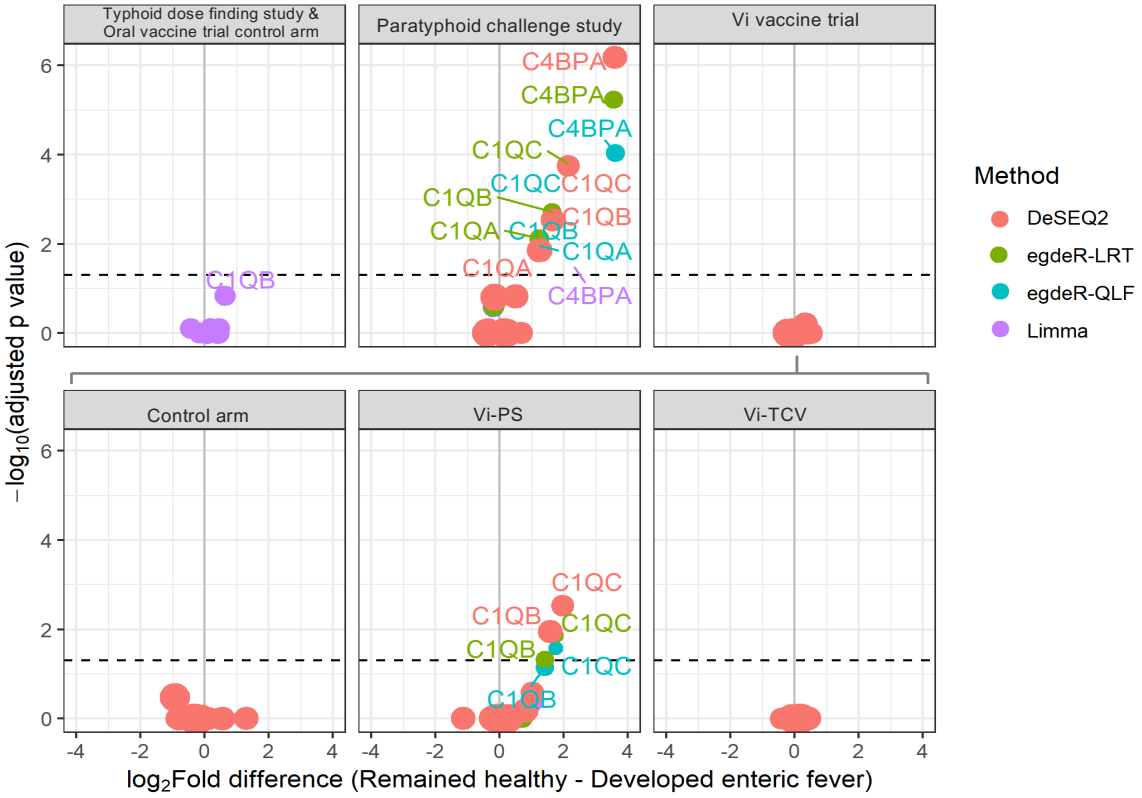
Table 5.2: Genes differentially expressed at baseline between those who developed enteric fever and those who remained healthy. P values are adjusted using a Benjamin-Hochberg correction. A positive $\log_2$(fold difference) indicates that the gene was more highly expressed in those who remained healthy.

Method	Group	Gene	Adjusted p value	$\log_2$(fold difference)
DeSEQ2	Paratyphoid challenge	*C4BPA* (Complement component 4 binding protein alpha)	0.0004	3.6
		SLC37A2 (Solute carrier family 37 member 2)	0.003	0.55
		C1QC (Complement C1q C chain)	0.04	2.1
	Vi-Polysaccharide	*GLDC* (Glycine decarboxylase)	0.01	-2.1
edgeR-likelihood ratio test	Paratyphoid challenge	C4BPA	0.004	3.6
		SLC37A2	0.009	0.5
		C1QC	0.03	2.1

	Vi-Polysaccharide	GLDC	0.01	-2.1
		ISG15 (ISG15 ubiquitin-like modifier)	0.02	2.3
		IFI27 (Interferon alpha inducible protein 27)	0.02	3.1
edgeR quasi-likelihood F-tests	Paratyphoid challenge	SLC37A2	0.03	0.5
		C4BPA	0.03	3.6
		C1QC	0.04	2.1
	Vi-Polysaccharide	GLDC	0.01	-2.1
Limma-VOOM	Vi vaccine trial (all groups)	ZNF208 (Zinc finger protein 208)	0.02	0.94

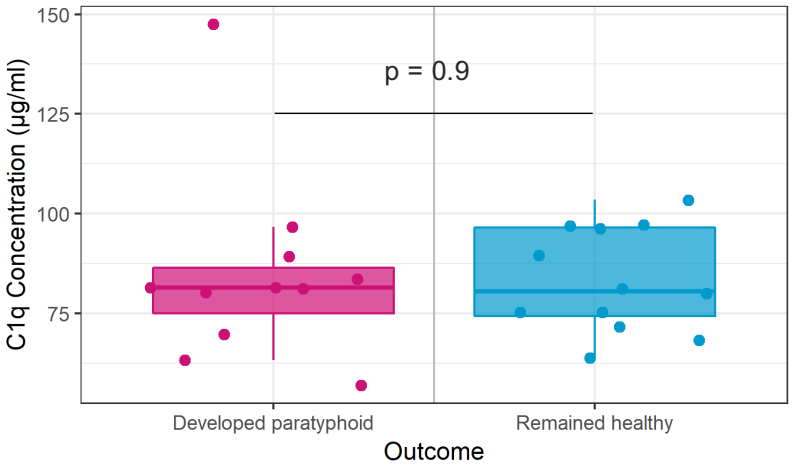
To further explore the association between complement and outcome in the paratyphoid challenge study, genes were subset to those annotated as complement related (25 genes) and p values re-adjusted based on the smaller number of variates (Figure 5.1). In the paratyphoid challenge group, in addition to *C1QC*, C1q chains *C1QA* and *C1QB* were also expressed at higher levels in those who remained healthy post-challenge. A similar pattern was observed in those who received the Vi-polysaccharide vaccine and remained healthy after typhoid challenge. Furthermore, in the two cohorts profiled by microarray, there was a trend towards higher *C1QB* expression in those who did not develop typhoid.

Figure 5.1: Log_2Fold difference in baseline complement gene expression between those who remained healthy and those who developed enteric fever, against the -log_{10}(Benjamin-Hochberg adjusted p-value) for each group. Points are coloured by the method of assessing differential expression. The dotted horizontal line indicates the threshold p = 0.05, after adjusting for multiple testing on complement related genes only.



To investigate whether C1q was associated with protection at the protein level, C1q concentration was measured in the baseline plasma from a subset of participants (n = 23) undergoing paratyphoid challenge. However, no significant difference was seen between those who did and did not develop paratyphoid fever (p = 0.9 by Mann–Whitney–Wilcoxon test, Figure 5.2), and correlation between RNA expression and protein levels was poor (Figure S5.3).

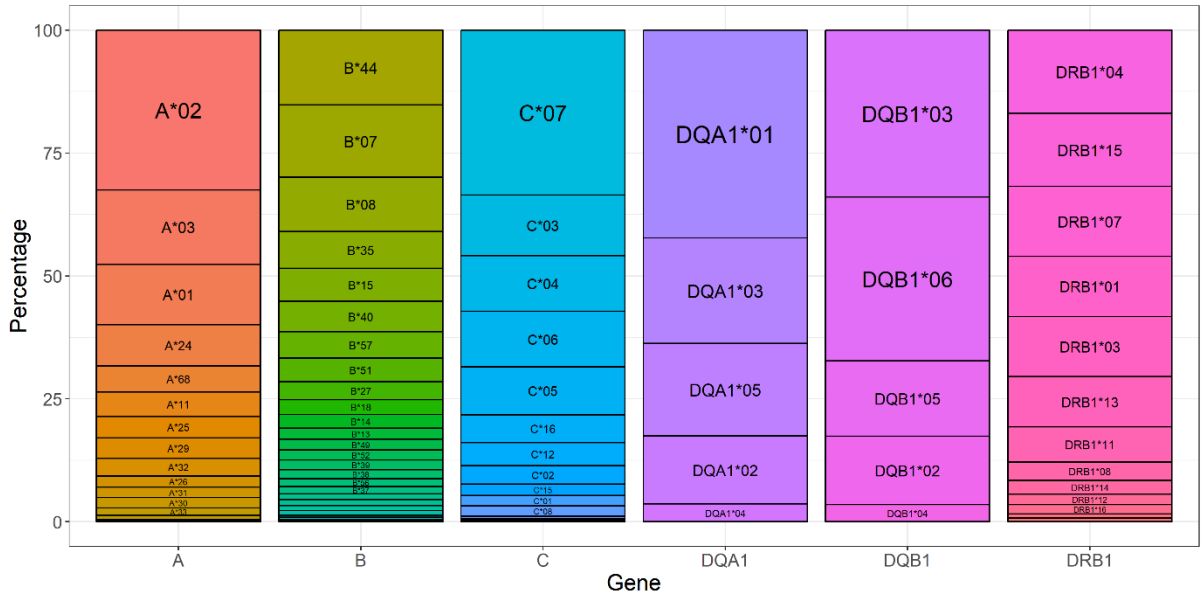
Figure 5.2: Baseline concentration of plasma C1q in participants undergoing paratyphoid challenge, plotted by outcome of challenge. Plasma samples from the paratyphoid dose finding study were diluted 1:100,000, and C1q quantified in duplicate using a sandwich ELISA. A Mann–Whitney–Wilcoxon test was used to calculate the p value shown.



5.3.2 Participants with HLA-type HLA-B*27:05 are more susceptible to enteric fever

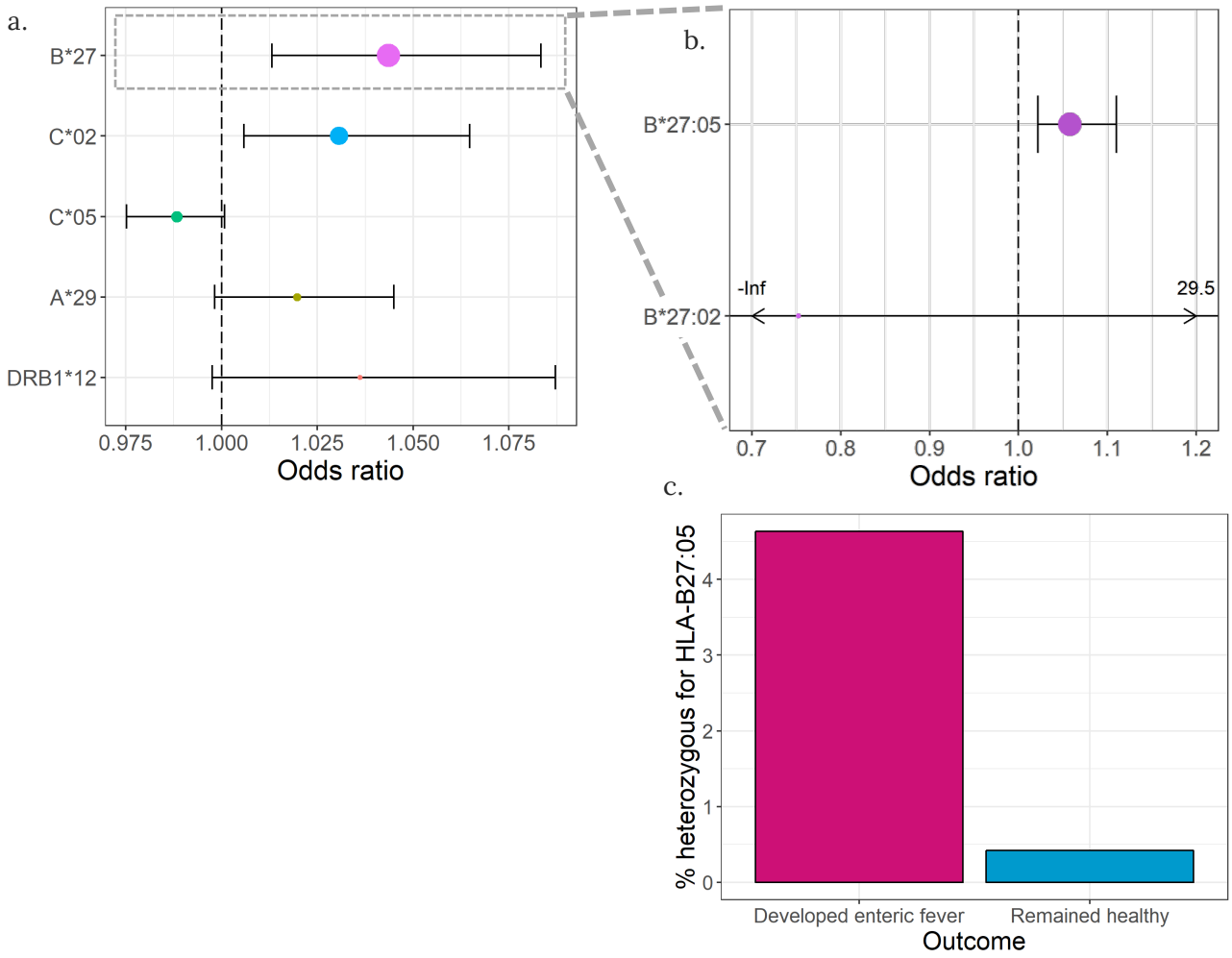
HLA types were either imputed from genotype data using SNP2HLA (Jia et al. 2013) (typhoid dose finding study and typhoid oral vaccine trial), or estimated from RNA-sequencing data using HISAT-genotype (Kim et al. 2018) (paratyphoid dose finding and Vi vaccine trial). As a validation of the method, for a subset of participants (n = 10) HLA types were estimated separately using data from two time points, and were found to have high reproducibility (Figure S5.4). Thus, HLA types were estimated for all participants from the baseline transcriptome. The relative frequency of each HLA type (1 digit resolution) in our cohort is summarised in Figure 5.3.

Figure 5.3: Relative frequency of each HLA type at a resolution of 1 digit for HLA-A, HLA-B, HLA-C, HLA-DQA1, HLA-DQB1 and HLA-DRB1 in our entire combined cohort, including participants from the typhoid dose finding study, typhoid oral vaccine trial, typhoid Vi vaccine trial, and paratyphoid dose finding study



A logistic regression model using vaccine and dose as covariates was used to assess association between HLA type and outcome of challenge. As this analysis was exploratory, all reported p values are not adjusted for multiple testing. HLA-B*27 and HLA-C*02 were most associated with susceptibility to infection ($p = 0.01$ and $p = 0.03$ respectively, Figure 5.4a), particularly HLA-B*27:05 ($p = 0.006$, Figure 5.4b and 4c).

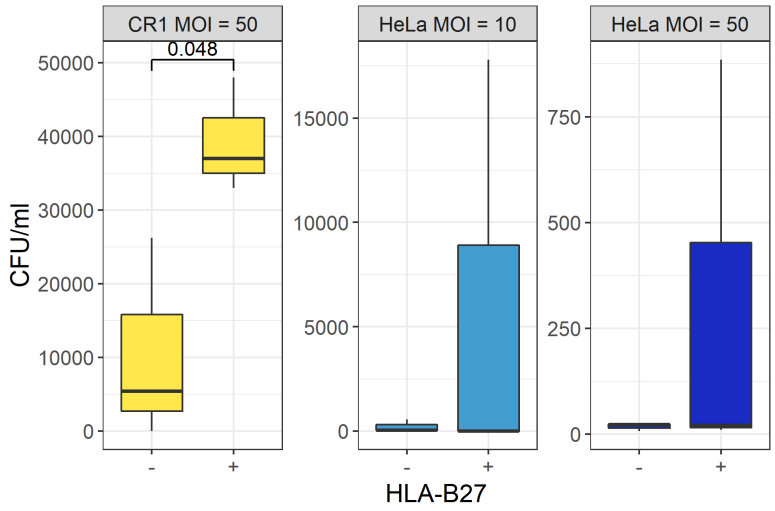
Figure 5.4: a. Odds ratios (odds ratio >1 indicates association with susceptibility and <1 with resistance) and 95% confidence intervals for HLA types with p value < 0.1 and frequency > 3 at a resolution of 1 digit. The size of the point reflects the significance of the association.
b. Odds ratios for the two B*27 sub-types at a resolution of 2 digits with 95% confidence intervals (off the scale for B*27:02 due to extremely wide confidence intervals). The size of the point reflects the significance of the association.
c. Percentage of participants heterozygous (dosage > 25%) for HLA-B*27:05 amongst those who went on to develop enteric fever and those who remained healthy



To assess whether a single cohort was driving the relationship between these variants and outcome, each challenge study was excluded in turn from the logistic regression model. The odds ratio for B*27:05 and C*02 remained stable at 1.05-1.07 and 1.02-1.07 respectively. When assessing each study individually, the p value for B*27:05 was only below 0.1 for the typhoid oral vaccine and typhoid Vi vaccine trials, the two cohorts with the greatest sample sizes (n = 67 and n = 102 respectively, compared with n = 32 and n = 26 in the other studies).

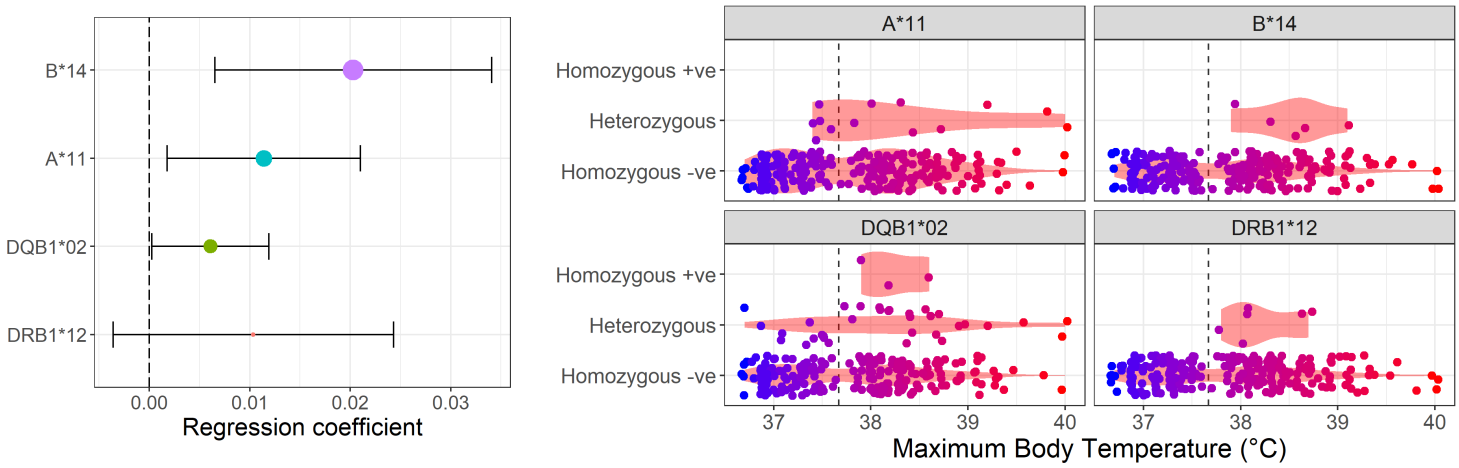
To further explore the relationship between HLA-B*27:05 and enteric fever susceptibility, HLA-B*27:05-positive and negative C1R and HeLa cells were infected with *S.* Typhi. One hour post-infection gentamycin was added to kill extracellular bacteria, and 24 hours post-infection lysed cells were plated to enumerate live intracellular bacteria. In the C1R lymphoblast cells, bacterial survival was higher in those expressing HLA-B*27:05 ($p = 0.048$ by t-test). At both an MOI of 50 and 10, mean intracellular survival was higher in HeLa cells expressing HLA-B*27:05; however variability was high (Figure 5.5).

Figure 5.5: Colony forming units per ml recovered from C1R and HeLa cells infected with *S.* Typhi Quailes strain, in the presence or absence of HLA-B*27 expression, 24 hours post-infection. Parent and HLA-B*27:05$^+$ cells were seeded in a 96 well plate at a density of 100,000 cells per well, and infected with *S.* Typhi Quailes strain at an MOI of 0 or 10 in triplicate. After one hour gentamycin was added to kill extracellular bacteria. 24 hours post-inoculation cells were lysed using 1% Triton-X100, and lysates serially diluted and plated onto tryptone soya agar. Colonies were counted following overnight incubation at 37°C. P values smaller than 0.05 (t-test) are indicated.



Interestingly, despite having been previously linked to protection against complicated typhoid fever (Dharmana et al. 2002), there was a trend in our cohort towards HLA-DRB1*12 being associated with infection susceptibility (p = 0.09, logistic regression). To assess whether in those who were diagnosed, HLA-DRB1*12 was associated with protection against more severe disease, maximum body temperature was used as a proxy for severity. HLA types B*14, A*11, and DQB*02 were associated with higher maximum body temperatures with HLA-DRB1*12 trending towards an association (p = 0.1, linear regression; Figure 5.6).

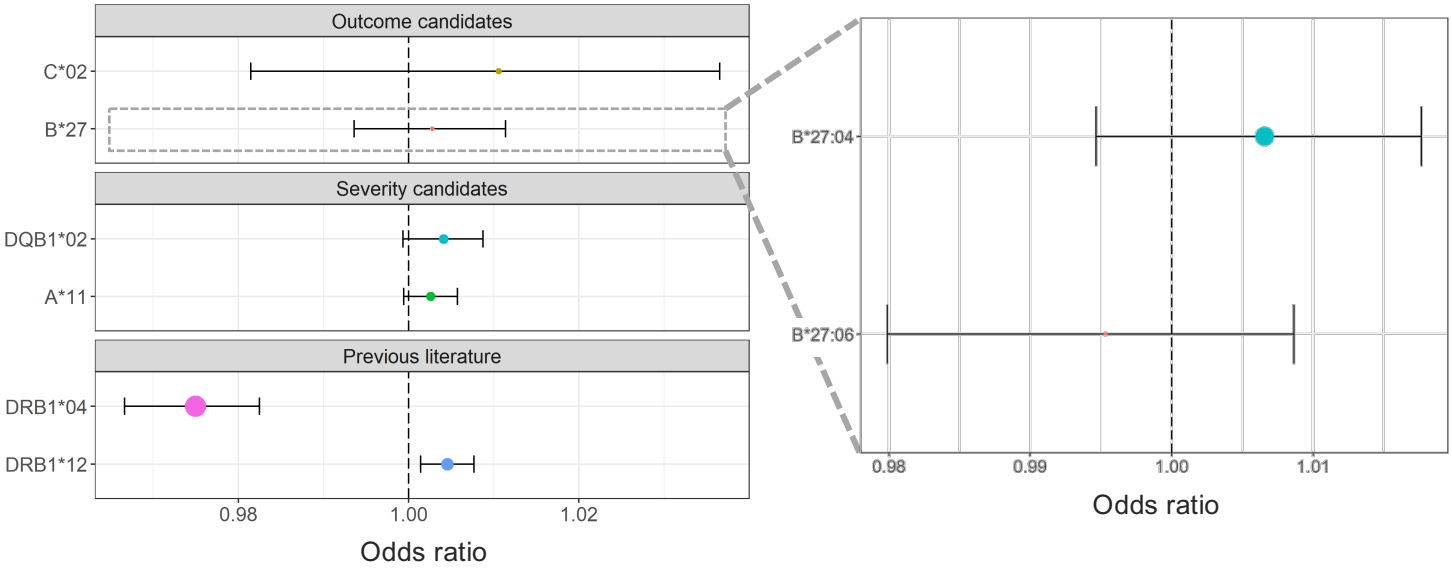
Figure 5.6 a. Regression coefficients for association with maximum body temperature, for three HLA types with p < 0.05 and frequency >3, and HLA-DRB12 type of interest HLA-DRB1*12. The size of the point reflects the significance of the association and the error bars 95% confidence intervals.
b. Maximum body temperature for each participant who developed enteric fever by HLA type (homozygous negative (dosage < 25%), heterozygous (25% < dosage < 75%) or homozygous positive (dosage > 75%)). As maximum body temperature followed a bimodal distribution, a dotted line is shown at the local minimum (37.6°C)



To explore whether these HLA types were associated with enteric fever susceptibility in an endemic setting, results were compared with that from a large case control study from Vietnam (Dunstan et al. 2014). HLA-B*27 and HLA-C*02, the alleles most associated with susceptibility in our challenge cohort, and A*11, and DQB*02, the alleles associated with disease severity (B*14 was not present in the Vietnamese cohort), all had an odds ratio > 1 but were not significantly associated with susceptibility in this study. HLA-DRB1*12, the allele previously identified as

protective against complicated typhoid fever, was associated with susceptibility (p = 0.004; logistic regression). HLA-B*27:05, the HLA sub-type which drove the association between HLA-B*27 and outcome in the challenge cohort, was not present in the Vietnam cohort (Figure 5.7).

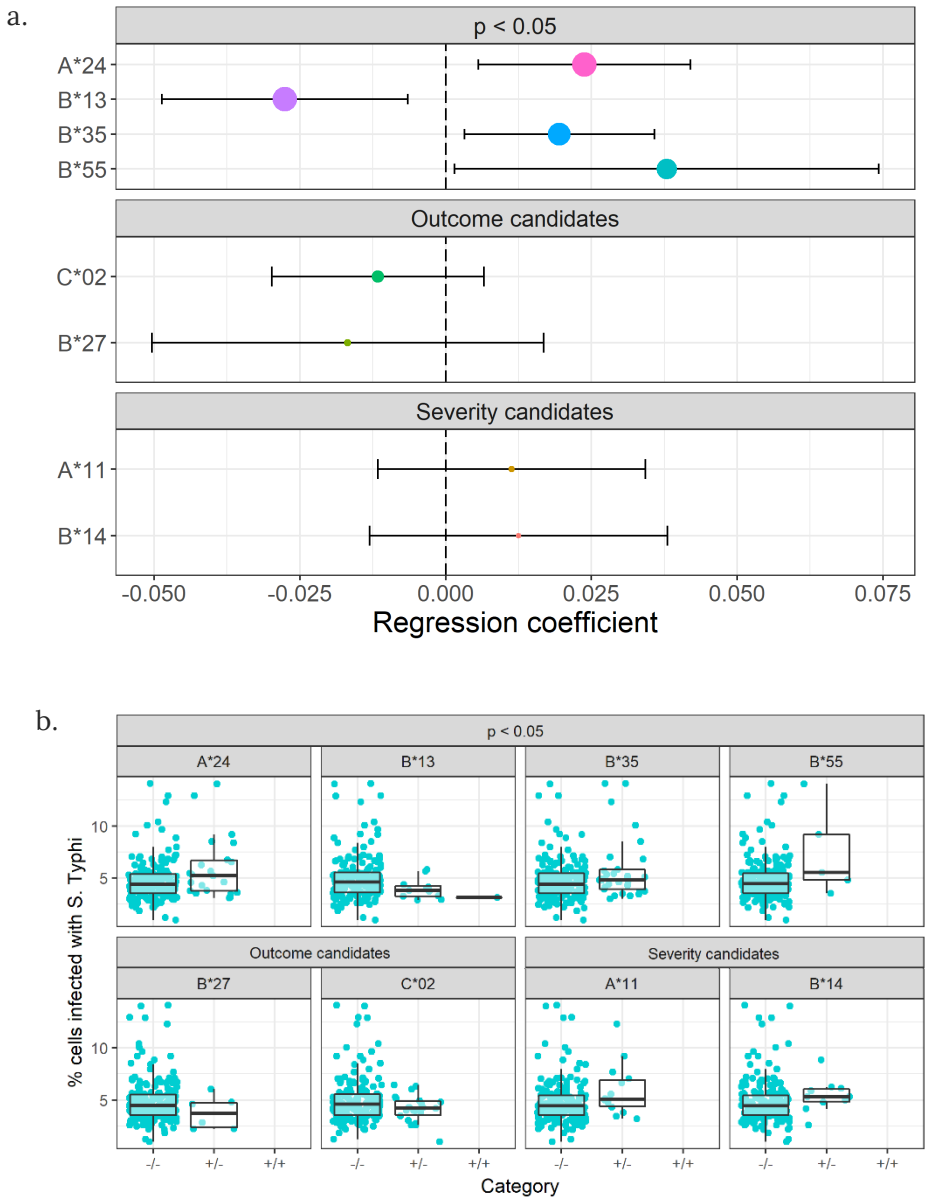
Figure 5.7: a. Odds ratios (odds ratio >1 indicates association with susceptibility and <1 with resistance) and 95% confidence intervals for the relationship between HLA type and enteric fever in a Vietnam case-control study. HLA types included are those associated with outcome of challenge and maximum body temperature in the enteric fever human challenge study, as well as the HLA type associated with resistance highlighted by this case-control study (HLA-DRB1*04) and a HLA type associated with resistance to severe disease highlighted by a separate study (HLA-DBR1*12). The size of the point reflects the significance of the association.
b. Odds ratios for the two B*27 sub-types in the Vietnam cohort at a resolution of 2 digits with 95% confidence intervals. The size of the point reflects the significance of the association.



Finally, we were interested to investigate the relationship between HLA type and expression of each gene in whole blood, as this may have a downstream effect on antigen presentation. 12 HLA types were associated (p < 0.05; linear regression) with the expression of their respective genes in the baseline transcriptome (Figure S5.5). The same 12 HLA types, as well as HLA-A*32, were significantly associated with gene expression 12 hours post-challenge. Furthermore, two HLA types, HLA-DQB1*04 and HLA-DQA*04, were associated with downregulation of their respective genes 12 hours post-challenge.

Publicly available data on the HLA type, baseline gene expression (as measured by microarray) and susceptibility to *S.* Typhi invasion of Hapmap human lymphoblastoid cell lines was used to build support for the HLA associations seen in the challenge study datasets. Matched *S.* Typhi *in vitro* infection susceptibility and HLA type was obtained for 204 cell lines, while matched gene expression and HLA type was obtained for 250 cell lines. Only HLA-A, B, C and DRB1 typing had been performed. Three HLA types (A*24, B*35, B*55) were associated with a higher percentage of infected cells when exposed to *S.* Typhi strain Ty2 for 3.5 hours, and one (B*13) with a lower percentage (Figure 5.8). While there was a trend towards greater bacterial invasion in those possessing the HLA types associated with maximum body temperature in the human challenge study, no such trend was seen in those associated with outcome of challenge. Furthermore, of the HLA types associated with gene expression in the human challenge study, only HLA-A*02, C*04, C*03 and C*17 were also significant ($p < 0.05$) and shared the same pattern of expression in the Hapmap human lymphoblastoid cell lines (Figure S5.6).

Figure 5.8: a. Regression coefficients with 95% confidence intervals for the relationship between HLA type and % of cells infected with *S.* Typhi. HLA types included are those with $p < 0.05$, and those associated with outcome of challenge and maximum body temperature in the enteric fever human challenge study. The size of the point reflects the significance of the association.
b. The proportion of cells infected with *S.* Typhi for each of the HLA types included in part a. Participants are grouped by whether they were homozygous negative, heterozygous, or homozygous positive.



5.3.3 Host polymorphisms are associated with variation in gene expression but not outcome of challenge

96 participants from the typhoid dose finding and typhoid oral vaccine trial were genotyped, of which one was excluded due to missing data (Figure S5.7). A further five participants were

excluded from association analysis on the basis of their outlying principal components, in order to prevent population structure from affecting the results (Figure S5.8). A logistic regression model incorporating challenge dose, vaccination status and sex as covariates was used to identify SNPs associated with outcome of challenge (Figure 5.9). Seven SNPs (Figure S5.9, Table 5.3) had a p value < 10^{-4}, of which three lay within genes *ADAM12*, *POT1-AS1* and *PRKCE* (Figure S5.10). Of the seven SNPs, five were present in the Vietnam case-control study; however none were significantly associated with enteric fever susceptibility in this study (p > 0.2).

Figure 5.9: Manhattan plot showing the significance (-$\log_{10}$(unadjusted p value)) of the relationship between each SNP and outcome of challenge for each chromosome. The dotted line indicates a p value of 10^{-4}.

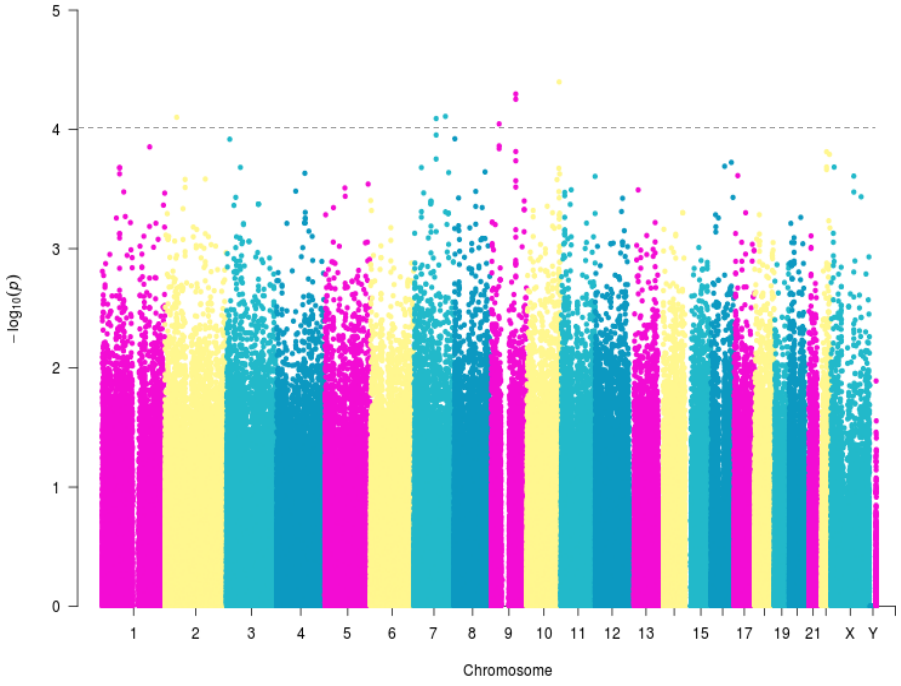


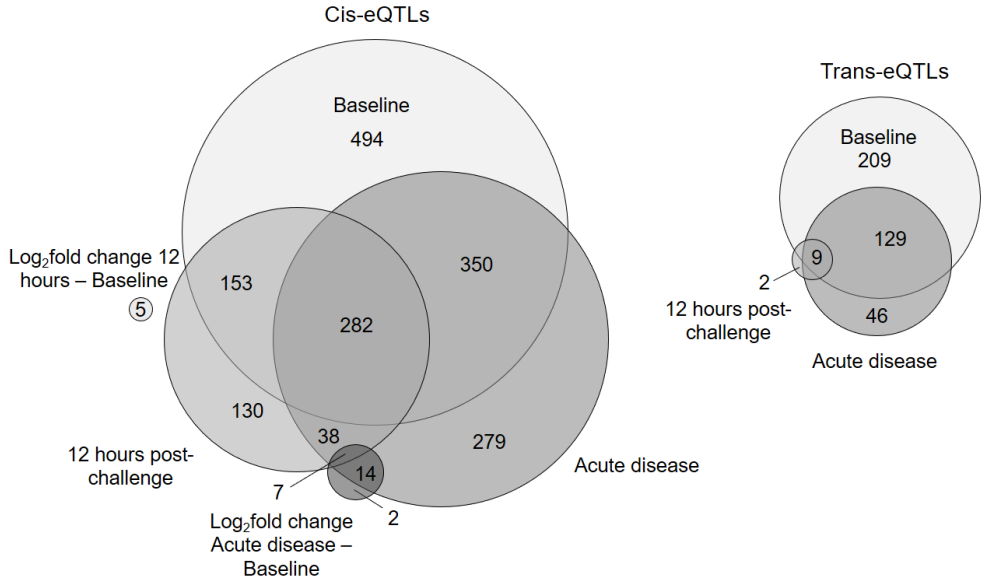
Table 5.3: SNPs with p < 0.0001 for association with outcome of challenge

SNP	Chromosome	p value	Gene
rs10901541	10	0.00004	Within intron of *ADAM12*
rs605323	9	0.00005	
rs543731	9	0.00006	
rs669064	7	0.00008	Within non-coding RNA antisense to *POT1*
rs2122113	2	0.00008	Within intron of *PRKCE*
rs181055	7	0.00008	
rs1328740	9	0.00009	

For participants in the typhoid dose finding study and typhoid oral vaccine study where both

transcriptomic and genotyping data was available, eQTL analysis was carried out to identify genetic variants related to gene expression. In order to elucidate relationships more likely to be of biological relevance, SNPs were filtered to those which have previously been significantly associated with gene expression in neutrophils, monocytes or B cells (Fairfax et al. 2012; Naranbhai et al. 2015). Cis-eQTLs were defined as being within 1kb of a gene, and trans-eQTLs as >1kb from a gene. After adjusting for multiple testing, 1,279, 610 and 970 cis-eQTLs were identified at baseline, 12 hours post-challenge, and during acute disease respectively (Table S5.1). There was a relatively large degree of overlap between eQTLs identified at each separate time point (Figure 5.10).

Figure 5.10: Overlap in significant eQTLs (FDR < 0.05) for each time point



Immune related genes (as defined by InnateDB (Breuer et al. 2013)) with significant (FDR < 0.05) eQTLs at these time points are shown in Table S5.2, including genes involved in TLR signalling (*CTSB, CTSK, CTSS, ITGB2, LY86, MAPK9*), complement (*CFB*, *C1QBP*), interferon signalling (*IRF5, KPNA1, PSMB8*) and antigen presentation (*CTSB, CTSS, HLA-E*). Despite not falling within 1kb, the majority of "trans"-eQTLs were within 1Mb of the associated gene, with the exception of a trans-eQTL for *PYHIN1* located on a different chromosome.

In addition to finding associations with gene expression at each time point, linear modelling was used to investigate relationships between genetic variants and transcriptional response to challenge. Five co-inherited gene variants ($R^2 = 1$) were significantly associated with a change in *SKIV2L* expression 12 hours-post challenge relative to baseline (Figure 5.11, $p = 2 \times 10^{-5}$, FDR = 0.03). Of these SNPs, only rs449643 falls in a coding region, causing an A>V amino acid change in the SKIV2L protein. Of the two homozygous participants which upregulated *SKIV2L*, one went on to develop typhoid fever and the other remained healthy. 29 cis-eQTLs were associated with a change in the expression of six genes during acute disease relative to baseline (Figure 5.12, FDR < 0.05): *CAT, ADCY3, PRUNE2, UROS, PSMB8* and a gene of uncharacterised function.

Figure 5.11: Log_2(fold change in SKIV2L gene expression at 12 hours post-challenge relative to baseline) for challenge participants of each genotype. As all associated SNPs were co-inherited ($R^2 = 1$) results are shown on one graph. The changes in gene expression for unchallenged controls (evening relative to morning) are shown for comparison, but were not genotyped.

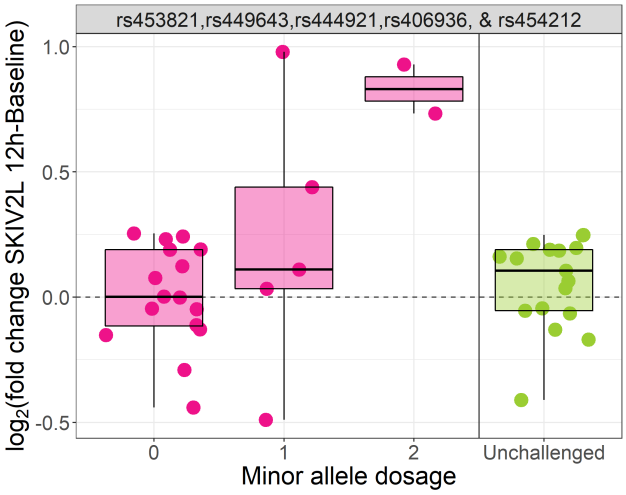
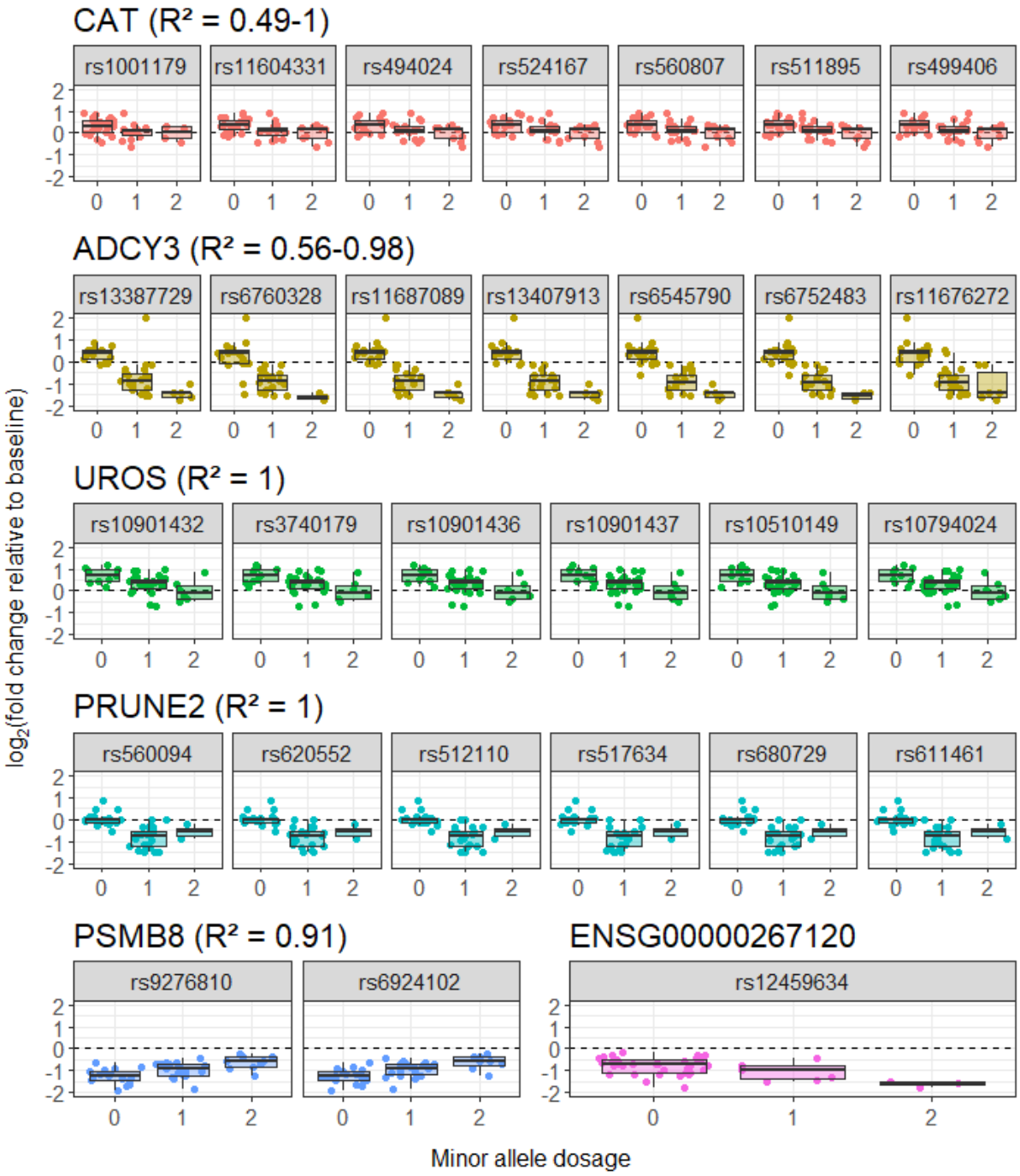


Figure 5.12: Log_2(fold change in gene expression in acute disease relative to baseline) for challenge participants of each genotype. R^2 values indicate the extent of linkage disequilibrium between significant cis-eQTLs for the same gene.



5.4 Discussion

5.4.1 Differences in the baseline transcriptome between outcome groups

In this chapter, genomics and transcriptomics were used to identify potential factors affecting host susceptibility to enteric fever. In the baseline transcriptome limma identified *ZNF208*, a zinc finger transcription factor, as being higher in those who did not go on to develop typhoid fever in the Vi vaccine trial. While its function is poorly defined, three SNPs in *ZNF208* have been associated with Hepatitis B susceptibility, supporting a potential immunological role, and one SNP with telomere length (H. Li et al. 2017).

DeSEQ2 and edgeR identified genes encoding C4BPA and C1QC, elements of the classical complement pathway, as being significantly higher in the baseline transcriptome of those who were challenged with *S.* Paratyphi A but did not go on to develop paratyphoid fever. Interestingly, this is in contrast to the enterotoxigenic *E. coli* challenge study where *C4BPA* was higher at baseline in those who developed symptomatic infection (Yang et al. 2016). As a candidate for involvement in susceptibility to paratyphoid, the classical complement pathway is highly biologically plausible. Firstly, whereas *S.* Typhi possesses a Vi polysaccharide capsule which prevents complement deposition (Wangdi et al. 2014; Wilson et al. 2011), *S.* Paratyphi A does not. Although C1q is a component of the classical antibody-dependent cascade, C1q has been found to directly bind *S.* Typhimurium LPS in an antibody-independent manner (Roumenina et al. 2008). Secondly, *C1qa* knockout mice have been found to be more susceptible to *S.* Typhimurium infection (Warren et al. 2002). Furthermore, polymorphisms in the C1q locus in humans have been found to relate to levels of serum C1q, suggesting that genetic variability at this locus could have a feasible effect on phenotype (Martens et al. 2009). Our observation of a trend towards higher *C1*q chain expression in those who received the Vi-polysaccharide vaccine and did not develop typhoid fever after challenge could be attributable to antibody-dependent complement-mediated killing. Another potential explanation is that in the presence

of Vi-antibody, bacteria may be under selection pressure to downregulate synthesis of the Vi capsule, leaving them more vulnerable to complement.

Despite this plausibility, limma, the most stringent of the differential expression methods, did not find any differences in baseline expression between those who did and did not develop paratyphoid fever. Furthermore, no difference in plasma C1q between outcome groups was observed, and correlation between C1q mRNAs and protein was poor. However, given that less than half of variation in protein abundance can be explained by mRNA levels within a cell, it is unsurprising that secreted plasma protein does not agree with whole blood mRNA sequencing (Vogel and Marcotte 2012), particularly as the C1q chain mRNAs were expressed at relatively low levels. Relevantly, although C1q mRNAs are expressed by both monocytes and macrophages, C1q protein is only produced following differentiation into macrophages (Kaul and Loos 2001).

5.4.2 Association between HLA types, challenge outcome and fever

Of the HLA-types, HLA-B*27, in particular HLA-B*27:05, was most significantly associated with susceptibility to infection. HLA-B*27:05 mis-folds in the endoplasmic reticulum, inducing the unfolded protein response, and has been linked with both reactive arthritis following Gram-negative bacterial infection (Antoniou et al. 2019), and ankylosing spondylitis (Khan 2013a). When infected *in vitro* with *S.* Typhimurium, both monocyte-like U937 Cells and epithelial HeLa cells transfected with HLA-B*27:05 have previously been found to exhibit higher levels of intracellular replication (Antoniou et al. 2019; Penttinen et al. 2004). Here we have replicated this result in C1R cells infected with *S.* Typhi Quailes strain, demonstrating this phenomenon is not serovar-specific. Previously, this enhanced replication was abrogated when HLA-B*27 was stabilised by fusion with beta-2-microglobulin (Antoniou et al. 2019). Although the exact mechanism is unknown, the unfolded protein response appears to create a favourable environment for *S.* Typhimurium, the presence of HLA-B*27 increasing its expression of SPI-2

genes (Ge, He, and Granfors 2012) and causing it to replicate at the cell periphery (Antoniou et al. 2019). Using publicly available data, we found no association between HLA-B*27 and percentage of lymphoblastoid cells infected with *S.* Typhi following *in vitro* infection. However, this is consistent with previous literature suggesting that while replication is higher in HLA-B*27^{+} cells, invasion is not affected (Pelliniemi et al. 1997). Furthermore, whereas the above experiments were carried out on transfected isogenic cell lines, the HapMap lymphoblastoid cell lines each have a different genetic background. HLA-B*27:05 was not present in the Vietnam case-control study population. However HLA-B27*04, the variant most associated with ankylosing spondylitis in Asian populations, had a positive odds ratio (Khan 2013b).

While HLA-DRB1*04:05 has been previously linked to enteric fever resistance (Dunstan et al. 2014), here the frequency in challenge study participants was too low to assess a relationship with challenge outcome. HLA-DRB1*12 however, previously associated with protection against complicated disease (Dharmana et al. 2002), was common enough in our cohorts to observe a trend towards association with susceptibility. HLA-DRB1*12 was also associated with susceptibility in the Vietnam case-control study. To investigate whether HLA-DRB1*12 might be related with attenuated disease, linear modelling was used to assess the relationship with maximum body temperature amongst those who developed typhoid fever. However, HLA-DRB1*12 once again trended towards susceptibility rather than resistance. A major limitation of this analysis was that no participants in the human challenge study met the criteria for "complicated" typhoid fever in the original study, which included intestinal perforation, shock and coma. We also investigated whether any HLA types were related to the gene expression of their respective genes. HLA-DQB*02 for example, which was associated with severity, was also associated with lower expression of HLA-DQB. As MHC class II genes are primarily regulated at the transcriptional level (Handunnetthi et al. 2010), this could have a marked effect on the presentation of phagocytosed antigens. However, the same pattern of expression was not observed in the lymphoblastoid cell lines.

5.4.3 Association between SNPs and outcome

The SNP most associated with susceptibility to enteric fever lay in the intronic region of ADAM12, a cell-surface metalloprotease which mediates shedding of extracellular domains from transmembrane proteins (Zhou et al. 2013). In epithelial cells, *ADAM12* mRNA is upregulated in response to TNF-α. Furthermore, overexpression of *ADAM12* results in increased secretion of neutrophil chemoattractants CXCL1 and IL8 in an EGFR dependent manner (Estrella et al. 2009). ADAM12 is also involved in regulation of T cell differentiation, its knockdown resulting in increased secretion of Th17 cytokines (Zhou et al. 2013). While not significant following a Bonferroni correction, one intronic SNP in *ADAM12* has been previously linked to mild influenza infection (Garcia-Etxebarria et al. 2015) ($p = 4 \times 10^{-8}$), and another with childhood ear infection (Tian et al. 2017) ($p = 9 \times 10^{-6}$). Despite not lying in a coding region, intronic SNPs may either be linked to a causative coding SNP, or themselves affect gene expression through splicing or transcription factor binding (Cooper 2010). However, the association between this SNP and enteric fever susceptibility was not validated in the Vietnam case-control study.

5.4.4 Association between host genetics and transcriptional response to challenge

eQTL analysis was carried out to identify genetic variants associated with variation in gene expression and transcriptional responses to challenge. Cis-eQTLs associated with the expression of immune genes were identified at baseline, 12 hours post-challenge and during acute disease, including genes involved in antigen presentation, TLR signalling, interferon-signalling and complement. There was marked overlap in cis-eQTLs between each time point, suggesting that many of these associations exist irrespective of enteric fever challenge. Therefore, analysis was carried out to identify SNPs associated with changes in gene expression relative to baseline. Five co-inherited SNPs within the *SKIV2L* gene were associated with upregulation of *SKIV2L* in two participants 12 hours post-challenge. The *SKIV2L* gene lies within

the MHC class III region, and encodes an RNA helicase which regulates RIG-I-like receptor-mediated antiviral responses (Eckard et al. 2014). Mutations in *SKIV2L* give rise to Tricho-hepato-enteric syndrome, resulting in chronic diarrhoea and immunodeficiency (Fabre et al. 2013). However, a rise in *SKIV2L* did not appear to be protective against disease. During acute disease, 29 cis-eQTLs were associated with changes in six genes relative to baseline. These included two eQTLs associated with the extent of *PSMB8* downregulation, which is located in the MHC class II region and encodes a subunit of the proteasome (Arimochi et al. 2016). This polymorphism could therefore affect the presentation of bacterial peptides to T cells.

5.4.5 Conclusion

This chapter represents the first time a genetic study has been carried out on human challenge study participants in order to investigate infection susceptibility and transcriptional responses. However, the major limitation on the analyses performed in this chapter is lack of statistical power. As tests were carried out on many SNPs, genes, and HLA types, the false discovery rate will have been high. In order to detect a SNP with a minor allele frequency of 0.1 and odds ratio of 1.1 with 80% power, almost 2000 participants would have been necessary (Johnson and Abecasis 2017). Therefore in the absence of any SNPs with very high odds ratios in our cohort, we were vastly underpowered to detect significant SNPs at a genome wide level. Differences in the baseline transcriptome between those who developed enteric fever and those who remained healthy were significant after adjusting for multiple testing. However, these results were not replicable using different tests and were not validated at the protein level. eQTLs were significant after adjusting for multiple testing, but as of yet no validation cohort is available. Thus, in order to make any conclusions with confidence, further work will need to be done to validate the hypotheses generated from this chapter, using either further *in vitro* experiments or independent validation cohorts.

Chapter 6 | Conclusions and limitations

This thesis aimed to better characterise early host response to enteric fever, identify genetic risk factors for infection, and find novel correlates of severity. A data-driven approach has been used to address these aims, taking advantage of transcriptomics, multiplex cytokine assays and genotyping. In the following chapter the questions set out in the introduction will be discussed in light of the findings from the previous three chapters.

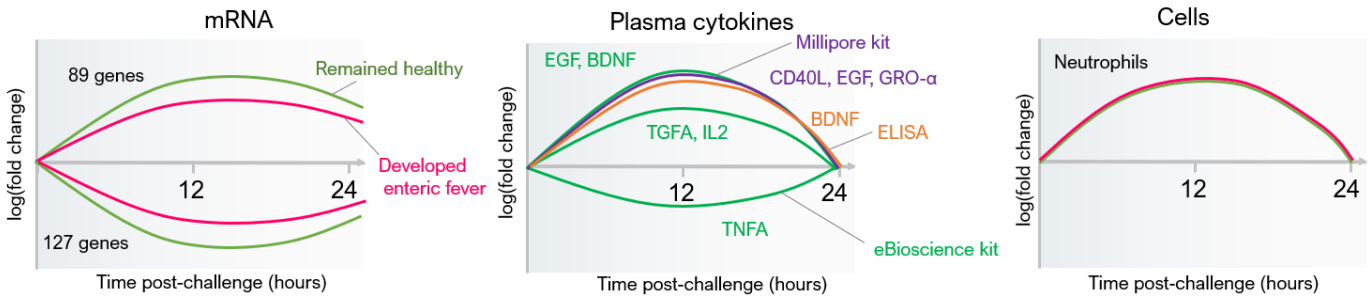
6.1 Is there a detectable early host response to enteric fever?

It has previously been demonstrated that 12 hours after challenge with *S.* Typhi Quailes strain, CD40L, fractalkine, GROα, IL1RA, EGF, and VEGF are raised relative to negative control participants (Blohmke et al. 2016). Here using the same multiplex assay manufacturer for analysis of an independent cohort, we find CD40L, GROα, IL1RA and EGF to be raised after challenge , and using a different assay manufacturer TGF-α and BDNF to be raised also. We find a rise in estimated neutrophil fraction and marked transcriptional perturbation in the blood, with upregulation of genes such as *LYST, MAFF, ST3GAL6* and *PTGR1* (summary in Figure 6.1). A similar response was observed following challenge with *S.* Paratyphi A. Five co-inherited SNPs were correlated with early changes in expression of *SKIV2L*, an RNA helicase which dampens down IFN-β response to RIG-I ligands (Eckard et al. 2014).

The presence of a detectable host response regardless of infection outcome raises the question of which point in disease pathogenesis a participant is committed to development of disease. From the data presented here, it appears co-administration of bicarbonate has been successful in neutralising the stomach acid, and the bacteria are consistently capable of reaching the intestine without being killed. This is supported by the observation that stool shedding occurs in both those who develop disease and those who do not (Gibani, Voysey, et al. 2019). What is not clear is whether *S.* Typhi successfully invades the epithelium in both groups; perturbation of the peripheral blood plasma cytokine profile and transcriptome could arise either due to

migration of activated cells from the intestinal mucosa, or from direct interactions between *S.* Typhi and cells in the peripheral blood during primary bacteraemia. Here we used blood transcriptional data from patients with inflammatory bowel disease to investigate how inflammation in the gut can modulate peripheral gene expression. The correlation between changes in the transcriptome 12 hours post-challenge and differences between inflammatory bowel disease and healthy controls would suggest migration of activated cells into the blood; however, the use of inflammatory bowel disease as a proxy for intestinal inflammation during enteric fever is limited in that whereas *S.* Typhi is thought to invade the terminal ileum, ulcerative colitis affects the colon, and Crohn's disease any area along the length of the digestive tract (M'Koma 2013). However as the terminal ileum is the most commonly affected area in Crohn's disease, specifically limiting Crohn's disease patients to those with terminal ileitis may provide a better model (Goulart et al. 2016). The detection of *S.* Typhi DNA in the blood of some non-diagnosed individuals early after infection on the other hand supports a more direct interaction (Darton et al. 2017). Given that even tooth brushing has been reported to give rise transient bacteraemia, asymptomatic bacteraemia early after typhoid challenge could be surprisingly common (Bhanji et al. 2002; Lockhart et al. 2008).

Figure 6.1: Schematic of the early response to enteric fever challenge. 12 hours post-challenge 89 genes are upregulated and 127 downregulated relative to unchallenged controls, with a greater magnitude of response in those who remain healthy. By eBioscience kit there is a rise in EGF, BDNF (FDR < 0.05), TGFA and IL2, and a drop in TNF ($p < 0.05$) in the plasma. By Millipore kit there is a rise in plasma CD40L, EGF and GRO-α, and by ELISA a rise in BDNF. Deconvolution of transcriptional data suggests a peak in neutrophils. Figure by A Barton.



This study was considerably limited by the effect of circadian rhythms. In the case of the transcriptomics analyses, although publicly available data from unchallenged participants were integrated into the analysis and batch corrected, this was carried out on a different microarray platform which may have differing sensitivity to changes in certain genes. Furthermore, the "differences of differences" comparison introduces a second level of random variation, and resulted in loss of statistical power due to lack of pairing. While this issue could be resolved by challenging participants both in the morning and evening, this is impractical due to the sample processing carried out on baseline samples. Another approach would be to take a blood sample the evening before a participant is challenged, so their response could be normalised to their own circadian rhythm. Although cytokines in control plasma were profiled for comparison, sample collection for the bicarbonate sub-study took place 3-4 years before collection of the typhoid toxin study, and therefore protein degradation may have occurred (De Jager et al. 2009).

A limitation to the multiplex cytokine assays identified here was the lack of consistency between kits produced by different manufacturers. While EGF, BDNF and to a lesser extent IL6, IL2, IL15, IL23, TGFA, IL7 and PDGF-BB were raised 12 hours post-challenge in one assay, sCD40L, GRO-α, EGF and CD40L were raised in another. This phenomenon has been observed previously (Berthoud et al. 2011), and we therefore recommend that changes in cytokines be validated by an ELISA optimised for the detection of a single cytokine.

Finally, the transcriptomics data was confounded by changes in cell composition, rather than wholly reflecting transcriptional upregulation and downregulation of genes. This in turn hinders the identification of eQTLs. Here we have attempted to address this limitation by comparing post-challenge changes to that of a homogenous cell line stimulated with *S.* Typhi. However in future this could be addressed by sorting cells before transcriptional profiling or carrying out single cell RNA-seq.

Future directions:

- Investigating whether knockdown of genes such as *LYST*, upregulated 12 hours post-challenge and in cells infected *in vitro* with *S.* Typhi, increases bacterial burden in infected cells
- Integration of RNA-seq data from unchallenged controls into the transcriptomic analysis
- qPCR for a selected subset of "challenge-specific" genes on both unchallenged control participants and a subset of challenged participants for further validation

6.2 Where does the early plasma cytokine response originate from?

It has yet to be elucidated whether the cytokine signal we describe 12 hours after enteric fever challenge arises directly from the intestinal mucosa during invasion; from leukocytes in the mucosa migrating into the peripheral blood; from peripheral leukocytes activated directly by *S.* Typhi during primary bacteraemia; or from the organs in which *S.* Typhi replicates during the incubation period. Here we have used a combination of publicly available gene expression data and *in vitro* stimulations of different cell populations to guide hypotheses on the origins of each early response cytokine (Figure 6.2). Answering this question in full would require *ex vivo* intracellular cytokine and surface marker staining of both blood cells and biopsies post-challenge. Flow cytometry could then be used to characterise cytokine secretion at a single cell level.

IL1RN and *CXCL1*, which were both raised 12 hours post-challenge in the typhoid dose finding study, and *TGFA*, which was upregulated in the typhoid toxin study, were consistently upregulated in the whole blood transcriptome 12 hours post-challenge. Normal expression of these cytokines in blood is highest in neutrophils (Monaco et al. 2019; Uhlén et al. 2015). Here

we found that secretion of IL1RA in response to *in vitro* stimulation with *S.* Typhi was neutrophil specific, while GRO-α (encoded by *CXCL1*) secretion was highest in neutrophils followed by monocytes. TGFA was raised in the supernatants of stimulated leukocytes as a whole but not in the supernatants of stimulated sub-populations, perhaps requiring cross-talk. Upregulation of these genes in the whole blood transcriptome could reflect either the migration of leukocytes from the inflamed intestinal mucosa into the blood, or direct interactions between peripheral leukocytes and S. Typhi. While *IL1RN* and *TGFA* are upregulated in the peripheral blood transcriptome of patients with inflammatory bowel disease, supporting the former scenario, *CXCL1* is upregulated in THP-1 cells in response to direct *S.* Typhi stimulation, supporting the latter.

Despite evidence of these cytokines rising in both the plasma and the blood transcriptome, correlation between the two at the level of the individual was poor. It may be that as well as expression by leukocytes, other tissues are secreting them; both *CXCL1* and *IL1RN* are upregulated in intestinal biopsies of patients with inflammatory bowel disease relative to controls (Verstockt et al. 2019), while *CXCL1* is upregulated in the liver of *S.* Typhimurium infected mice (Del Bel Belluz et al. 2016). Furthermore, GRO-α, IL1RA and TGFA were each secreted by intestinal organoids following *S.* Typhi infection.

Other cytokines raised in the plasma 12 hours post-challenge were unchanged the blood transcriptome, implying they were either released from pre-existing intracellular stores in response to infection, or secreted by other tissues into the blood. EGF and BDNF were elevated in the post-challenge plasma in the typhoid toxin study, and were both validated by independent methods. BDNF is produced by PBMCs in response to LPS (Cattaneo et al. 2016) and is raised in the plasma during influenza infection (Chiaretti et al. 2013). However it is primarily expressed in the cerebral cortex (Uhlén et al. 2015), and stored but not expressed by platelets in the blood (Fujimura et al. 2002). In response to *in vitro* infection of leukocytes with *S.* Typhi, BDNF was consistently but non-significantly raised in the supernatant in a dose

dependent manner. Nevertheless, *BDNF* mRNA is only lowly expressed in the blood (Monaco et al. 2019), was not upregulated in the peripheral blood 12 hours post-challenge, and is not upregulated in cells or biopsies stimulated with *S.* Typhi, or in inflammatory bowel disease. Thus, the rise may either be due to release of pre-synthesised BDNF by leukocytes or platelets, or increased secretion by the brain. *EGF* mRNA is highly expressed by the kidney and pancreas but low in the blood. Despite this, granulocytes secreted significant EGF in response to *S.* Typhi infection. *EGF* is also upregulated in the jejunum of *S.* Typhimurium infected mice and the colon of patients with ulcerative colitis, suggesting it may be intestinal in origin.

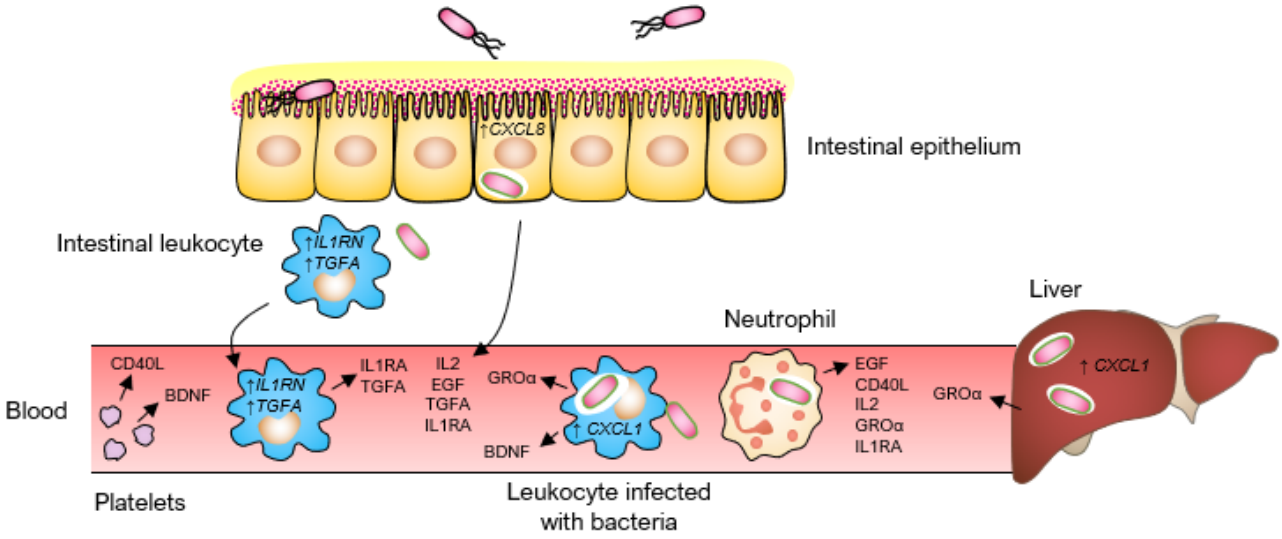
While IL2 and IL6 were determined to be somewhat raised in plasma 12 hours post-challenge using a multiplex cytokine assay from one manufacturer, this was not replicated using another manufacturer's assay. Conversely, a rise in secreted CD40L was detected using the second assay but not the other. In future work these discrepancies could be addressed by performing ELISAs for individual cytokines. *In vitro*, IL6 was produced by monocytes, granulocytes and lymphocytes following *S.* Typhi infection. IL2 and CD40L were secreted by granulocytes only, despite their mRNA expression in normal blood being highest in T cells (Monaco et al. 2019; Uhlén et al. 2015). CD40L is also released by activated platelets (Aoui et al. 2014; Mobarrez et al. 2015). Outside of the peripheral blood, IL2 expression is highest in the rectum and small intestine (Uhlén et al. 2015), and is secreted from the apical side of intestinal biopsies in response to *S.* Typhi infection. IL6 expression on the other hand was raised in intestinal biopsies from patients with inflammatory bowel disease. Thus, IL2 and IL6 could conceivably be intestinal in origin.

TNFA levels in plasma were observed to drop 12 hours post-challenge in the typhoid toxin study; however, this result was not replicated when the same samples were profiled using a different kit, or following *in vitro* cell stimulation. IL8, investigated as it was upregulated at an mRNA level in intestinal biopsies stimulated with *S.* Typhi and had previously been intensely

studied in the context of *Salmonella*-host interactions, did not significantly rise in the blood transcriptome or plasma.

Determining the sources of the cytokine signal would provide clues as to which cells and tissues *S.* Typhi interacts with early in infection, and therefore guide vaccine development. If the signature was wholly of intestinal origin, an oral live attenuated vaccine inducing robust mucosal IgA responses may offer greater protection, while if the signature originated from the peripheral blood a parenteral vaccine targeting invasive bacteria would be a more rational approach.

Figure 6.2: Model of the source of cytokines raised in the plasma 12 hours post-challenge. *IL1RN* and *TGFA* are transcriptionally upregulated in leukocytes in response to intestinal inflammation, while *CXCL1* is upregulated in response to direct infection. *CXCL1* is also upregulated in the liver during *S.* Typhimurium infection. Activated platelets release CD40L and BDNF, of which BDNF is also produced by infected leukocytes, and CD40L by infected granulocytes. *S.* Typhi infected granulocytes also release EGF, IL2, GRO-α and IL1RA. In response to infection, the intestinal mucosa itself transcriptionally upregulates *CXCL8* (as observed in *S.* Typhi infected biopsies), *EGF* and *IL1RN* (as observed in inflammatory bowel disease), and releases IL2 (as observed in *S.* Typhi infected biopsies), TGFA, IL1RA and GROα (as observed in *S.* Typhi infected organoids). Figure by A Barton.



Future directions:

- Carrying out *in vitro* stimulations using a lower MOI to investigate whether this gives a cytokine profile more reflective of that *in vivo*

- *Ex vivo* flow cytometry on cells stained for surface markers, homing markers and intracellular cytokines to characterise changes in cytokine profile at a single-cell level
- Use of mouse models or biopsies to assess the extent to which tissues such as the intestine, liver or spleen contribute to changes in plasma cytokines after challenge, and whether systemic dissemination is necessary to induce changes

6.3 Is it possible to predict who will develop enteric fever after exposure?

Finding early differences between those who do and do not develop disease after challenge would allow identification of important protective mechanisms, give clues as to which point in infection progression to acute disease is determined, and potentially act as a diagnostic biomarker following suspected exposure. While the human challenge model has an advantage over conventional case-control studies in that everyone is exposed to a highly similar agent, here we are limited by a small sample size. Furthermore, as human challenge studies predominantly take place in a Caucasian cohort, findings are not necessarily applicable to the population in endemic countries.

Genotyping revealed no SNPs significantly associated with outcome following correction for multiple testing. None of the most significant associations were replicated in an independent cohort. Although there were differences in the baseline transcriptome between outcome groups, these proved unreproducible between cohorts and differential expression methods. High baseline levels of *C1QC* were associated with protection following paratyphoid challenge, but at the protein level C1q was not significantly higher in those who did not develop disease. There were no major differences in early transcriptional responses to challenge, although the

magnitude of transcriptional perturbation appeared to be higher in those remaining healthy. While principle component analysis on the top 10 genes differentiating the two outcome groups gave distinct clustering, this was not reproducible. Significant eQTLs were identified at baseline and post-challenge, but it is not clear that any were associated with susceptibility.

Participants possessing HLA-B*27:05 were found to have a slightly higher odds ratio for development of disease. B cell-like C1R cells expressing HLA-B*27:05 were subsequently identified as having a higher bacterial burden following *in vitro* infection than control cells, suggesting susceptibility may be due to an intracellular environment which promotes *Salmonella* replication (Antoniou et al. 2019; Pelliniemi et al. 1997; Zhao et al. 2017) rather than altered antigen presentation. As HLA class I molecules are expressed throughout the body, the effect of HLA-B*27:05 on replication could have an effect on any stage of pathogenesis. However, HLA-B*27:05 is only found at a high frequency in Caucasian populations, and therefore is not a relevant genetic risk factor in endemic areas. However due to its association with reactive arthritis following *Salmonella* infection, in future volunteers for typhoid human challenge studies may be screened before enrolment. HLA-B*27:05 could also conceivably confer greater susceptibility to travel-related enteric fever infections or gastroenteritis.

Future directions:

- Genotyping is being carried out on the paratyphoid challenge cohort for eQTL validation
- Validation of the observation that transcriptional perturbation in greater in those who do not develop disease using a fourth cohort
- Investigating whether there are differences in the transcriptional response between HLA-B27^{+} and HLA-B27^{-} participants

6.4 What factors affect disease symptomology?

As challenge dose, strain and timing is controlled, human challenge models provide a unique opportunity to identify host determinants of severity. Several HLA types (HLA-B*14, HLA-A*11, HLA-DQB1*02) were associated with a greater maximum temperature during acute disease. While HLA-DRB1*12 has previously been associated with resistance to enteric fever (Dharmana et al. 2002), here we see the opposite trend. The relationship between HLA types and disease severity has been intensely studied in the context of dengue fever (Chaturvedi, Nagar, and Shrivastava 2006), and is presumed to be related to the presentation of T cell epitopes.

Increases in plasma cytokines IL1RA, MIG and IP10 were correlated with severity in acute disease. MIG and IP10 have previously been associated with severity in a range of diseases, including acute respiratory infections (Hayney et al. 2017), melioidosis (Lauw et al. 2000), tuberculosis (Hasan et al. 2009), hepatitis C infection (Esmon et al. 2011), and dengue fever (Ferreira et al. 2015). However, it is unclear whether these cytokines directly contribute to symptoms, as has been previously found for endogenous pyrogens IL1 and IL6. As biomarkers of disease severity, the combined rise in these cytokines could be used as an objective measure of symptom attenuation following vaccination, and therefore judge efficacy in the absence of sterilising immunity.

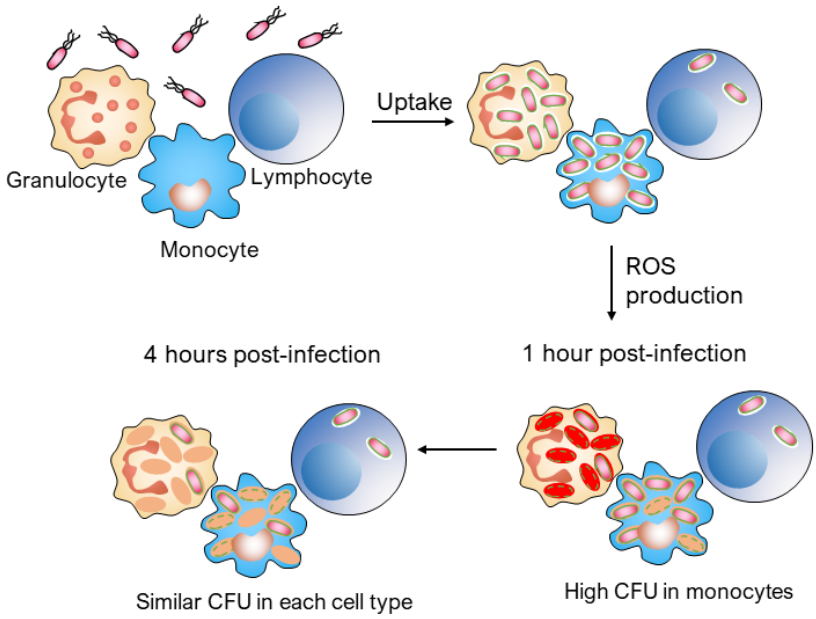
Future directions:

- Investigating whether the addition of recombinant MIG, IP10 or IL1RA increases bacterial infection *in vitro*, or if siRNA knockdown reduces it
- Identifying transcripts correlating with severity during acute disease, and whether any SNPs are correlated with these

6.5 What role do neutrophils play in early responses to *S.* Typhi?

Deconvolution of transcriptional data by Cibersort suggested a peak in neutrophils 12 hours post-challenge, consistent across different study cohorts and not occurring in the control dataset. The rise did not differ between those who did and did not develop disease, suggesting this response is not protective. Neutrophil chemoattractants GRO-α and IL8 were released by leukocytes in response to stimulation with *S.* Typhi in a dose-dependent manner, but only GRO-α was raised in the blood 12 hours post-challenge. As GRO-α was produced by granulocytes in response to *S.* Typhi infection, it is unclear whether the rise in GRO-α is mediating the rise in neutrophils or vice-versa. The lack of protection offered by a neutrophil response raises the question as to whether neutrophils are a viable intracellular niche for *S.* Typhi, contributing to its systemic dissemination. While infection of peripheral neutrophils could occur in the blood during primary bacteraemia, there is also evidence that neutrophils are able to reverse migrate from inflamed tissues back into the blood (Buckley et al. 2006; Hughes et al. 1997; Mathias et al. 2006). *S.* Typhi is typically thought to disseminate in monocytes, and indeed we found the intracellular survival of bacteria in monocytes to be very high one hour post-infection. Yet 4 hours post-infection, despite strong induction of reactive oxygen species in granulocytes, survival was equal between granulocytes and monocytes (Figure 6.3). It is possible that a sub-population of bacteria are able to adapt to the environment within granulocytes, which could be confirmed in future by longitudinal experiments of greater length and bacterial transcriptomics.

Figure 6.3: Model for the differences in uptake, surviving intracellular bacteria and reactive oxygen species observed between granulocytes, monocytes and lymphocytes following *in vitro S.* Typhi infection. Both granulocytes and monocytes have high bacterial uptake, while uptake by lymphocytes is low. In response to *S.* Typhi infection granulocytes produce high levels of ROS, while monocytes produce lower levels of ROS, resulting in higher CFU in the monocytes after one hour. However 3 hours later further killing has occurred in the monocytes but not in the granulocytes, resulting in equal bacterial survival at this time point. Figure by A Barton.



This analysis was limited in that peripheral blood counts were not available for the 12 hour post-challenge time point, preventing more direct confirmation of a rise in neutrophils. Although flow cytometry was used to phenotype neutrophils from a subset of challenge study participants, there were marked batch effects with unpredictable levels of activation. From this small subset of participants, a rise in the proportion of neutrophils in the peripheral leukocyte population was not obvious, and also likely to be subject to batch effects. As primary neutrophils are short-lived, long *in vitro* stimulation experiments were not feasible, but could be carried out on neutrophil-like cell lines such as HL-60. Finally, for practical purposes in vitro experiments were carried out on granulocytes separated by density, rather than pure neutrophils. Interactions between *Salmonella* and other granulocytes are as yet uncharacterised, but a major drop in eosinophils during acute disease has been observed (Waddington, Darton, Jones, et al. 2014).

Future directions:

- Peripheral blood counts early after challenge and in unchallenged controls to confirm the findings seen by deconvolution
- Longer incubations of *S.* Typhi with granulocytes or HL-60 cells following addition of gentamycin to assess longer term survival and adaptation, ideally with bacterial RNA-seq

6.6 Conclusion

While there have been a number of limitations encountered in the work outlined in this thesis, summarised in table 6.1, this thesis also represents a number of firsts in the field. Chapter 3 outlined the most detailed characterisation of the plasma cytokine response to *S.* Typhi infection made to date, both in terms of number of cytokines measured and number of timepoints. This is the first time the effect of the typhoid toxin on plasma cytokine responses has been investigated in humans *in vivo*; the first time the origin of the "early response" cytokines has been explored, and the first time MIG, IP10 and IL1RA have been identified as potential biomarkers of symptomology in acute disease, with evidence of attenuation by vaccination. BDNF was identified as a novel early response cytokine, and has not previously been studied in the context of gastrointestinal infection. This is the first time the transcriptional response to a bacterial pathogen has been characterised during the incubation period, with evidence of an early innate response which is greater in magnitude in those who remained healthy. We find that there may be an early rise in neutrophils in the blood, and that granulocytes can take up, change phenotype and produce ROS and cytokines in response to *S.* Typhi despite presence of the Vi capsule. This is the first time ROS production has been characterised simultaneously in three different cell populations, for a wide variety of *Salmonella* strains in the absence of antibody. From publicly available data, we hypothesise that

changes in the transcriptome and cytokine profile may plausibly be attributed to both host-pathogen interactions in the intestine and with peripheral blood leukocytes. To our knowledge this is the first time GWAS and eQTL analysis has been carried out in a human challenge model, and we identify HLA-B*27:05 not only as a risk factor for reactive arthritis, but for typhoid fever in a predominantly Caucasian population, by a mechanism which may be independent of antigen presentation.

Table 6.1: Summary of the limitations of the work carried out in this thesis

Chapter	Limitation	Potential solution
3	Results given by different multiplex cytokine assays are conflicting	Validation by ELISA
	Plasma samples for unchallenged controls were collected several years before the typhoid toxin study, and may have suffered from degradation	Collection of new plasma from unchallenged controls administered bicarbonate
	Self-reported symptom scores are subjective	Use of more objective measures of severity such as length of bacteraemia
	Lack of participants with complicated enteric fever in order to assess true markers of severe disease	Validate biomarkers of severity in field samples from patients with complicated enteric fever
	Assumption that changes in cytokines are independent of one another in statistical tests	Using unsupervised clustering to identify clusters of co-correlated cytokines, and identifying which clusters are up- and down-regulated
	Separation of cells into granulocytes, monocytes and lymphocytes before stimulation did not give 100% pure fractions and does not preserve interactions between different cell types	Intracellular cytokine staining
	In vitro stimulation of cells with *S.* Typhi appears to be more inflammatory than *in vivo* infection	Use of lower MOIs
	Unlike intestinal tissue, iPSC-derived organoids do not contain immune cells	Set up of a more complex co-culture system or use of *ex vivo* intestinal biopsies
	A significant rise in a cytokine or correlation with symptomology does not given direct evidence of an important role in disease or immunity	Knock-out of the genes encoding these cytokines in cell lines or a mouse model, or addition of recombinant cytokines
4	Transcriptional profiling of the unchallenged control cohort took place using a different platform, and unchallenged controls were not administered bicarbonate	Transcriptional profiling of challenged and unchallenged controls using the same technology, with unchallenged controls having been administered bicarbonate
	24 hour post-challenge samples were transcriptionally profiled in a different batch	Transcriptional profiling of a subset of baseline, 12 hour and 24 hour post-challenge samples in one batch

	Infection of cells with stained bacteria changed the distribution of cell MFIs but did not give discrete peaks, preventing the number of bacteria taken up per cell to be quantified. It was unknown whether bacteria were phagocytosed or attached to the cell surface, and there were differences in the intensity of bacterial staining between different strains and conditions	Confocal fluorescence microscopy Expression of GFP by the bacteria or use of conjugated anti-LPS antibodies to increase staining specificity Optimising staining for each strain so as to be the same intensity
	Difficultly validating whether neutrophils were activated in human challenge participants *ex vivo* due to staining batch effects	Fixing cells as soon as possible after collection and then staining as one batch once collection at different time points is complete Focusing on the comparison between challenged and unchallenged controls 12 hours post-challenge rather than comparing to baseline
	Changes in gene expression were compared to stimulated cell lines THP-1 and Hep2, which do not have the same characteristics as primary cells (for example, THP-1 lacks Fc gamma RIII), and were stimulated with an attenuated *S.* Typhi stain	Transcriptional profiling of primary leukocytes stimulated with Quailes strain *S.* Typhi, ideally separated into different cell subsets
	Changes in gene expression were compared to the transcriptome of IBD patients; in many cases of Crohn's and all cases of ulcerative colitis however disease is not localised to the terminal ileum, where *S.* Typhi is thought to invade	Limiting to patients with terminal ileitis Transcriptionally profiling blood samples from patients infected with non-invasive enteric pathogens such as *S.* Typhimurium
5	Small sample size and lack of a validation cohort	Genotyping a greater number of participants
	No validation of HLA typing by RNA-sequencing method	Genotyping a subset of participants for which there is also RNA-seq data available, allowing comparison between imputation by genotype and by RNA-seq
	Association of a locus or gene with outcome does not directly elucidate its function in disease	Knock-out of associated genes in cell lines or a mouse model

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Appendix I: Supplementary figures

Figure S3.1: Plasma Cytokine Samples. Plasma samples were taken and assayed at time points indicated by coloured boxes. Pre-diagnosis time points are highlighted in grey, post-diagnosis time points in pink, and samples excluded due to outlying principal components in blue.

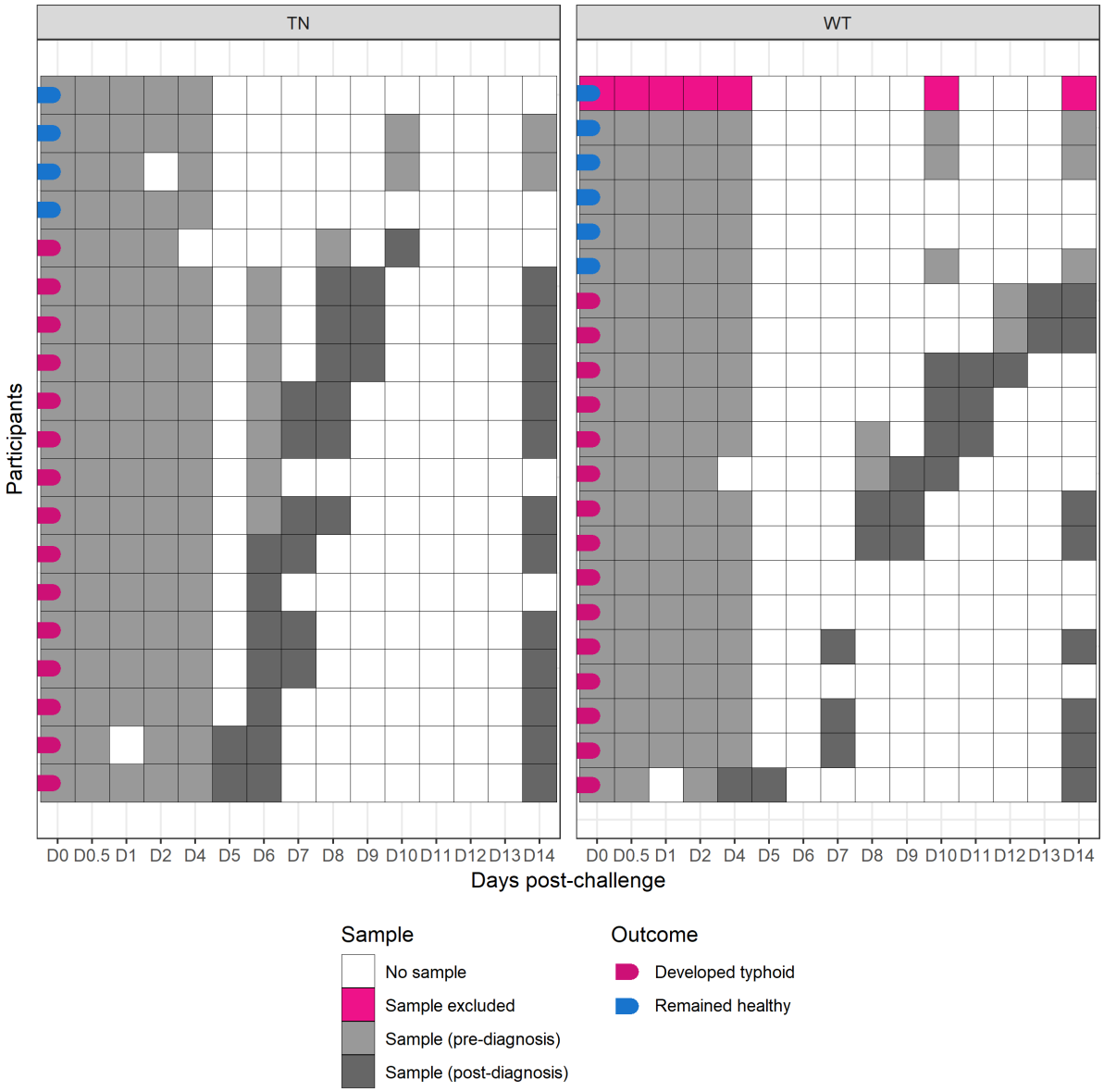


Figure S3.2: PC1 vs PC2 for a principal component analysis on the concentrations for each sample. Technical duplicates are shown as separate points. Samples are coloured by plate number, while the shape of each point represents challenge group.

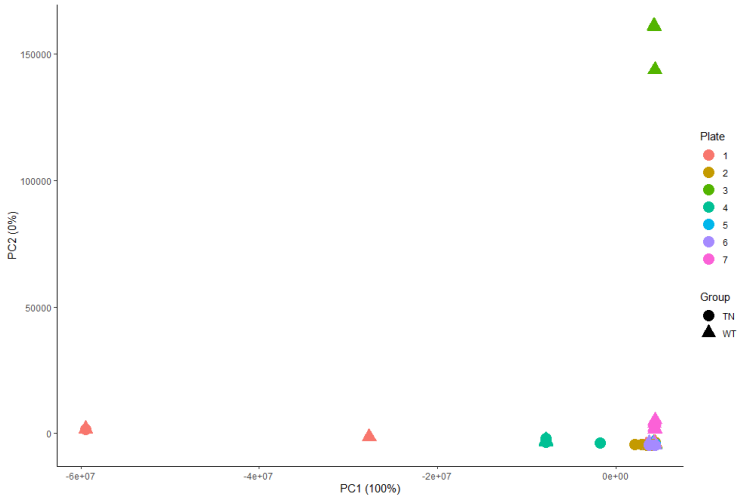


Figure S3.3: Distribution of median fluorescence intensities (MFIs) for each sample before and after quantile normalisation

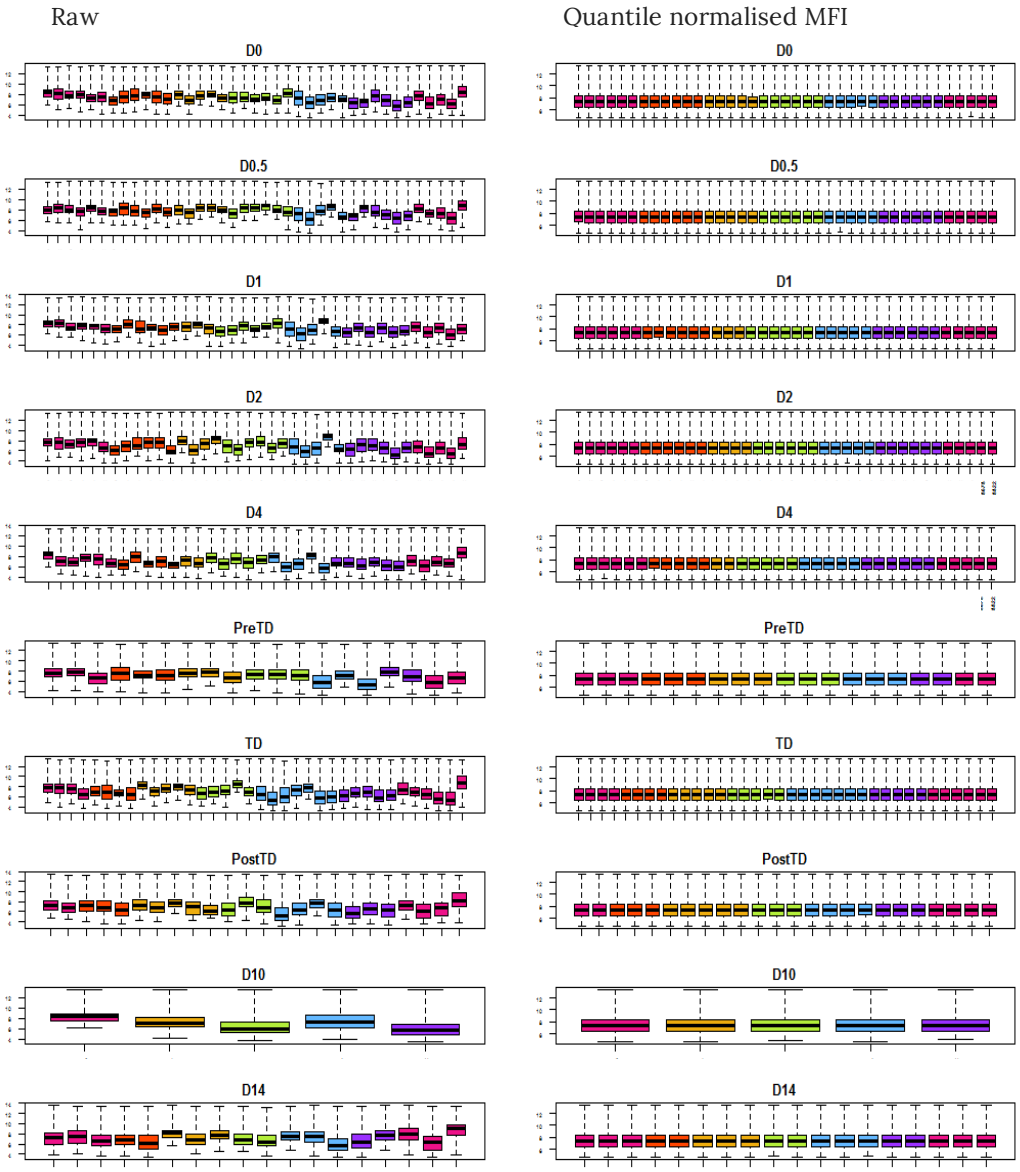


Figure S3.4: Heat maps by time point. Samples are clustered by Euclidean distance, and cytokines are ordered by the magnitude of their greatest logFC in MFI. Bars between the heat map and column dendrogram indicate outcome (maroon = diagnosed, blue = non-diagnosed) and challenge group (green = WT, yellow = TN)

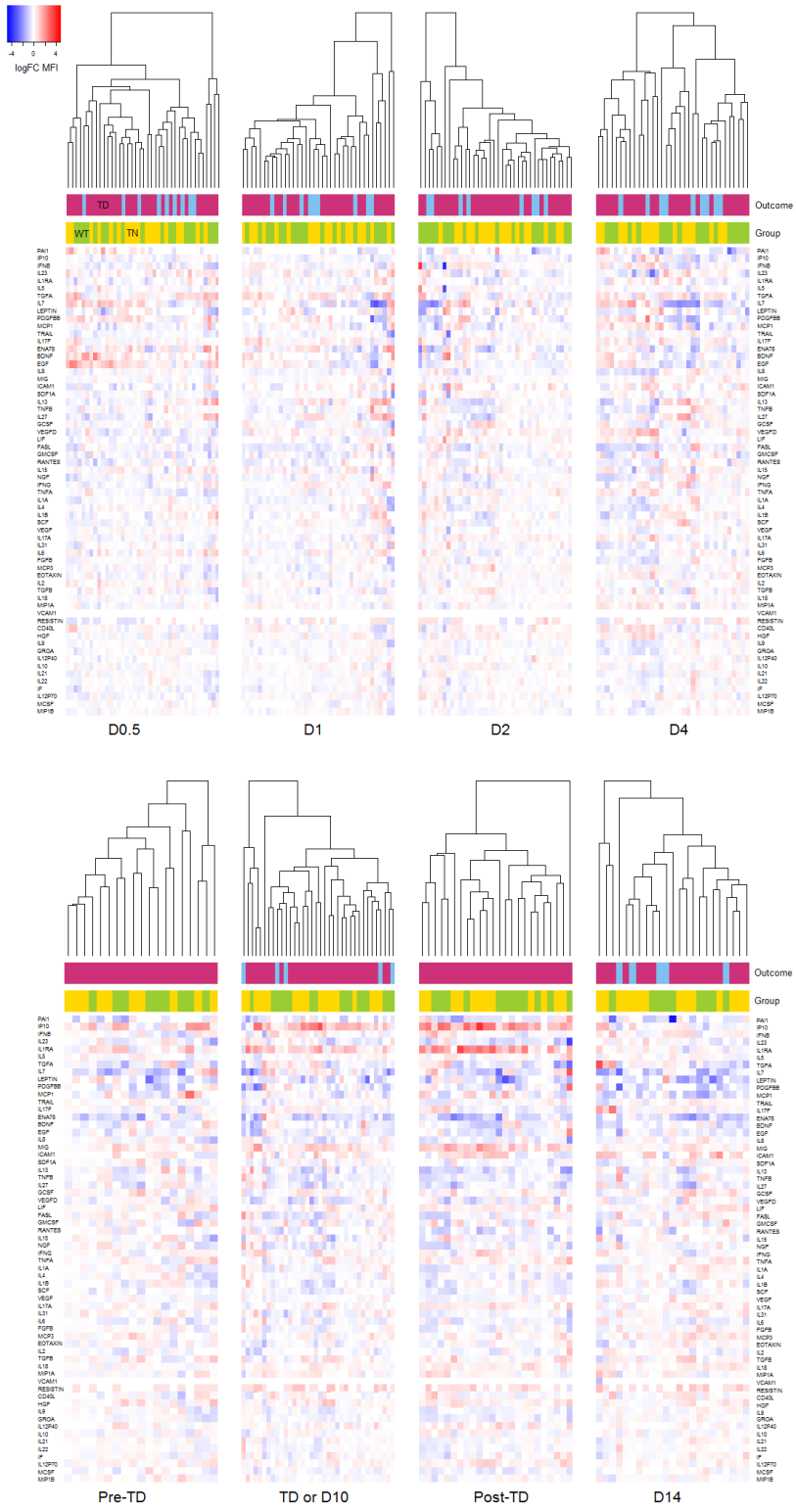


Figure S3.5: PCAs at each individual time point. Ellipses reflecting 95% confidence intervals are shown by outcome (pink = TD, blue = nTD) and group (yellow = TN, green = WT)

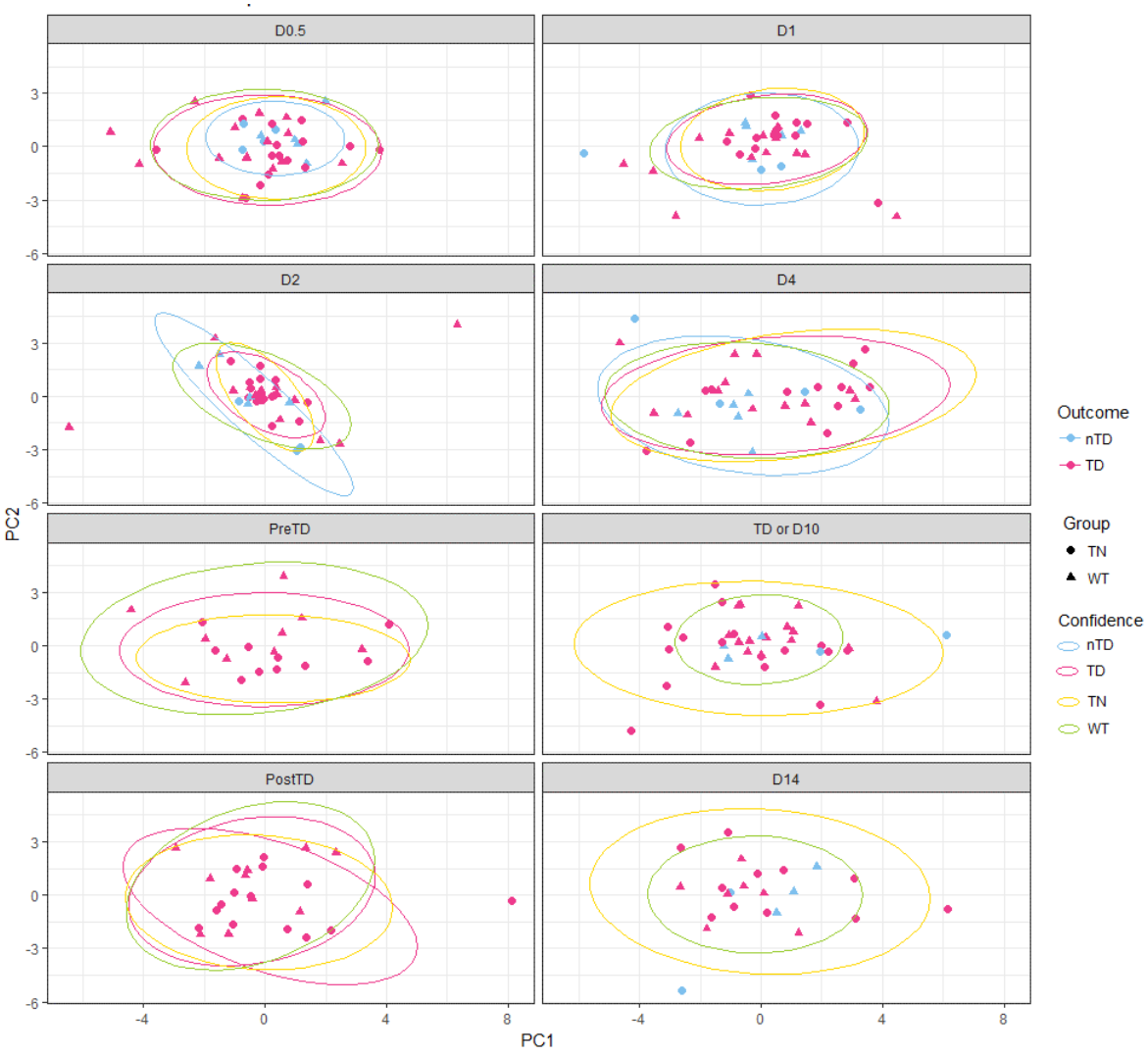


Figure S3.6: Concentration of IL-8 in the supernatants of leukocytes from one healthy donor stimulated with Quailes stain *S.* Typhi for 3 hours at MOIs 0, 0.1, 1, 10 and 100. Heparinised blood was collected from one healthy volunteer, and white blood cells isolated by red blood cell lysis and washing. White blood cells were seeded in a 96 well plate at 50,000 cells per well. A frozen stock of *S.* Typhi Quailes strain was thawed, washed and added to white blood cells at an MOI of 0.1, 1 , 10 or 100 in duplicate. Cells were stimulated with 100ng/ml *S.* Typhi LPS as a positive control and RPMI as a negative control. After one hour 100µg/ml gentamycin was added to kill extracellular bacteria. After a further two hours supernatants were harvested and spin filtered. IL-8 was quantified using a Human IL-8 ELISA Ready-SET-Go! eBioscience kit carried out according to manufacturer's instructions. Duplicates were averaged and concentrations calculated by fitting a four parameter logistic regression curve to the standard curve.

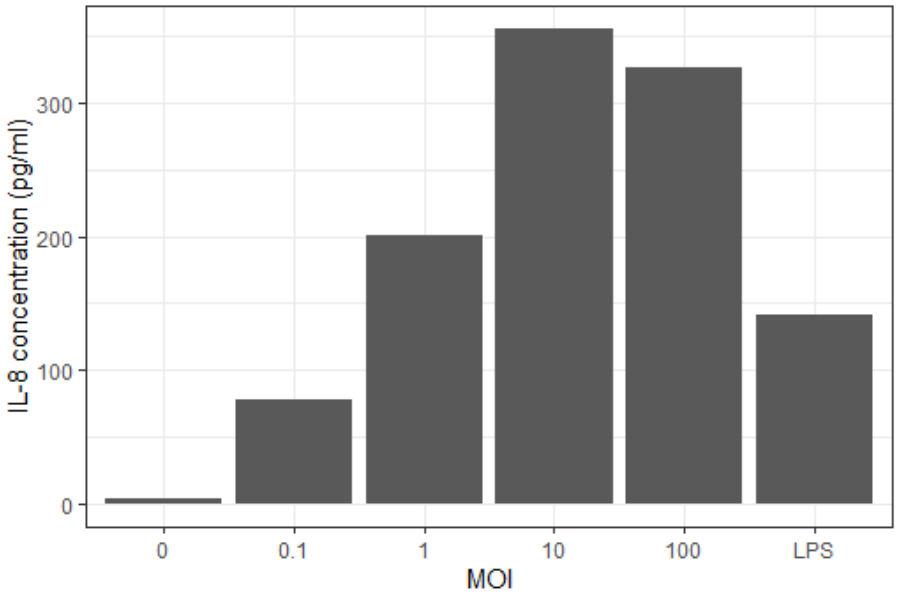


Figure S3.7: Heat maps showing the Pearson correlation coefficients (ρ) for the correlation between the logFC MFI of each cytokine relative to baseline and temperature, symptom score and C-Reactive Protein, overall and at individual time points. For each heat map cytokines are ordered by the ρ value for correlation across all time points. Correlations which are significant before adjusting for multiple testing are highlighted by blue asterisks (*** < 0.001, ** < 0.01, * < 0.05)

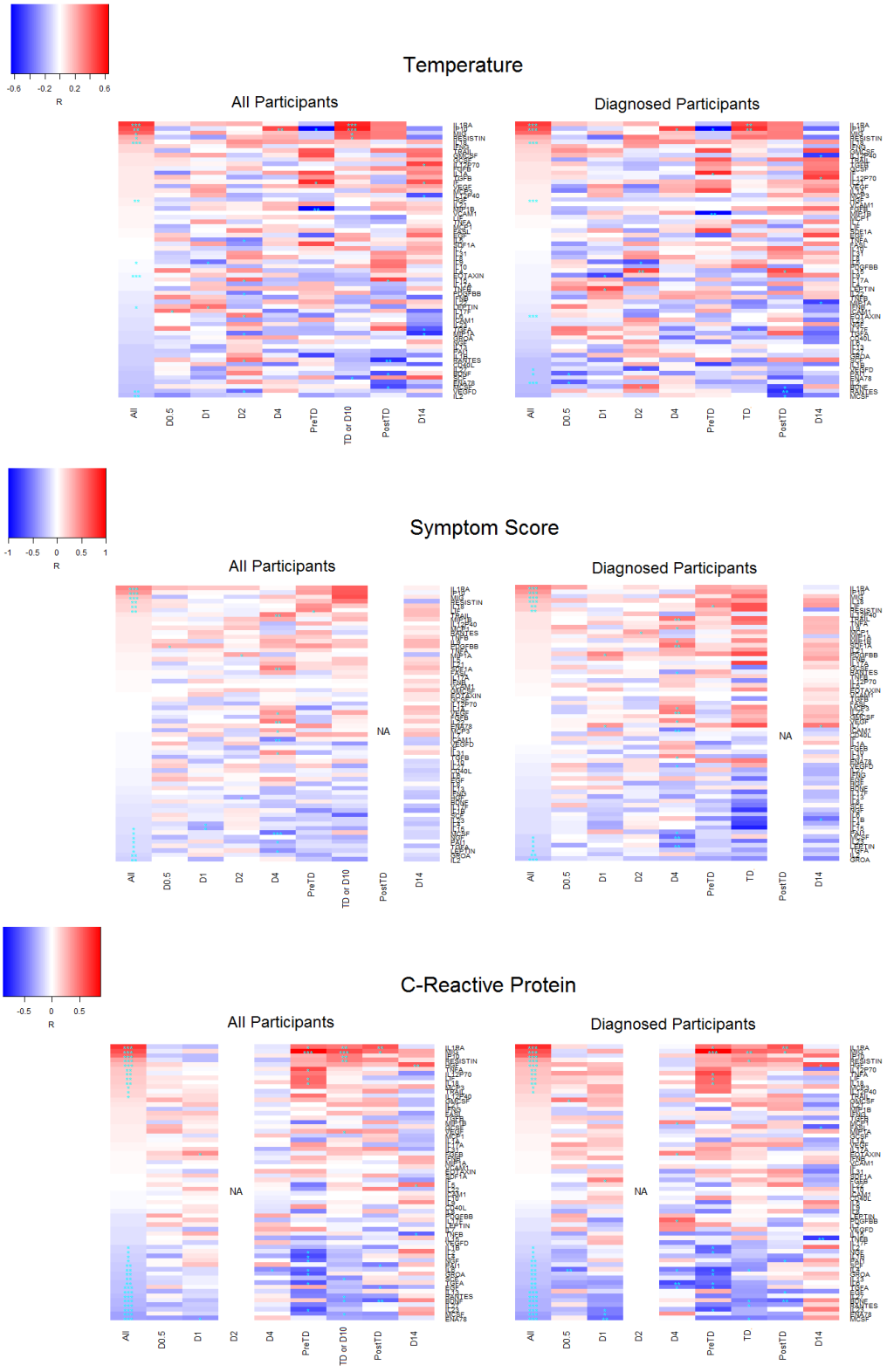


Figure S3.8: The $\log_2$(fold change) in MIG, IP-10 and IL1RA MFIs relative to baseline. Loess curves with 95% confidence intervals are shown for participants with severe typhoid (pink), mild/moderate typhoid (orange) and participants who stayed healthy (blue). Individual measurements from each participant are joined by grey lines. Time points are shown relative to the time of typhoid diagnosis.

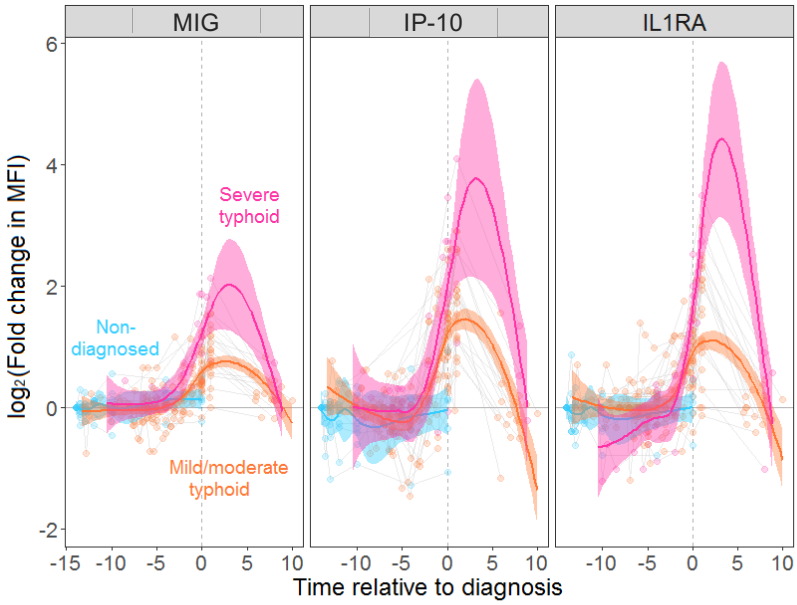


Figure S3.9: Correlation between IL1RA and IL1B. Black solid lines indicate the best fit line across all time points amongst non-diagnosed and diagnosed participants with 95% confidence intervals indicated by shading. Coloured dashed lines show best fit lines within individual time points. The indicated ρ and p values are for all groups and time points combined.

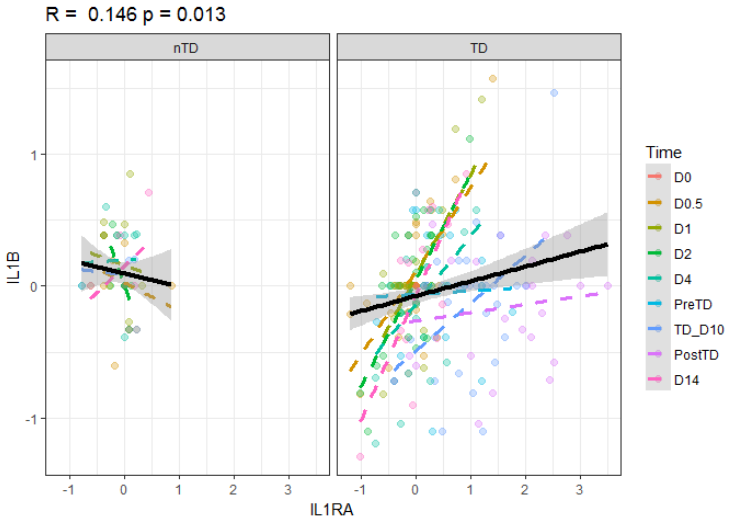


Figure 3.10: Correlation between IL1RA and other cytokines. Pearson correlation coefficients (ρ) are shown on a blue-red continuous scale. IL1B and IL1A, which directly compete with IL1RA, are highlighted. Asterisks indicate whether ρ is significantly different from 0 (p < 0.001 ***, p < 0.01 **, p < 0.05 *).

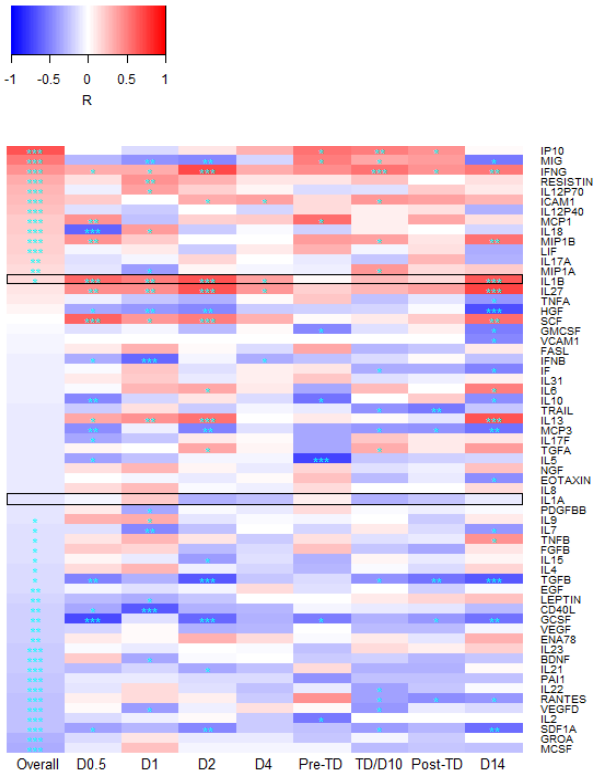


Figure S3.11: Heat maps showing the Pearson correlation coefficients (ρ) for the correlation between the logFC MFI of each cytokine at the given time point relative to baseline with temperature and C-Reactive Protein, overall and at individual time points. For each heat map cytokines are ordered by the R value for correlation across all time points. Correlations which are significant before adjusting for multiple testing are highlighted by blue asterisks (*** < 0.001, ** < 0.01, * < 0.05)

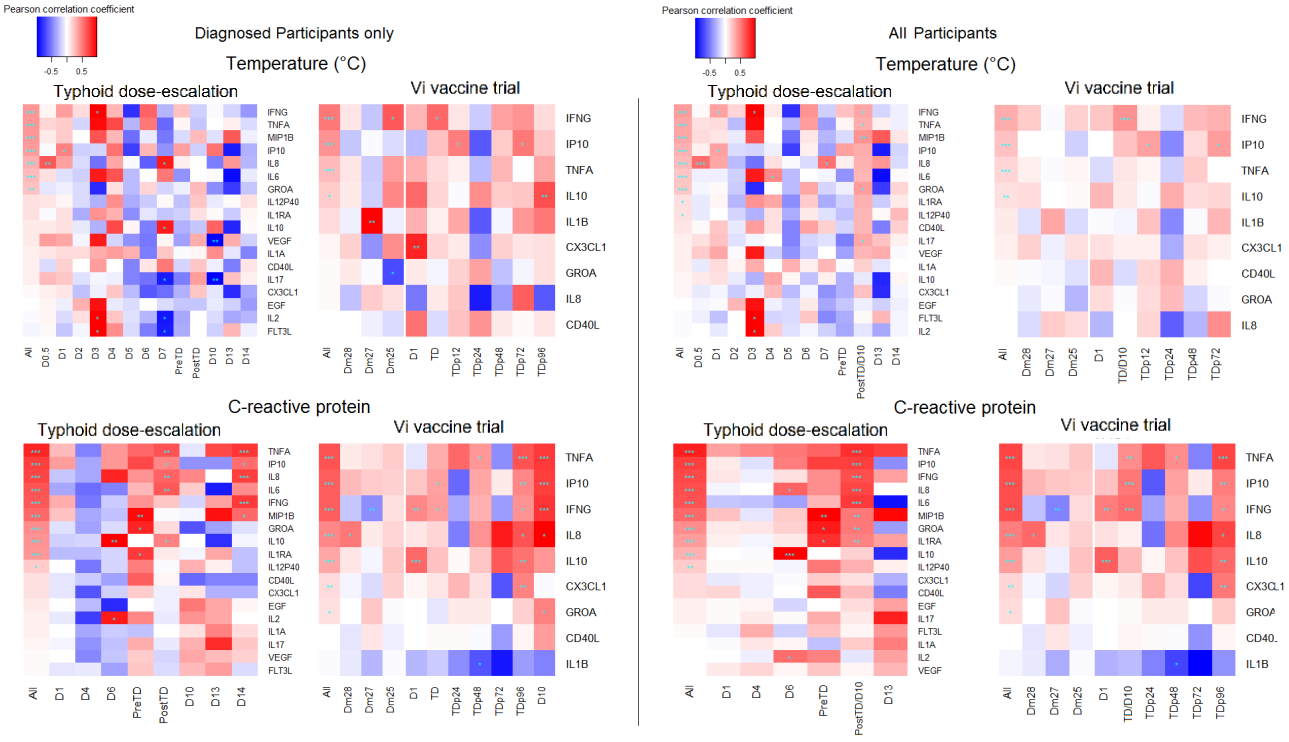


Figure S4.1: The distribution of RNA concentrations as measured by Nanodrop, and RNA integrity numbers (RIN) as measured by Agilent Bioanalyzer kits for the discovery and paratyphoid challenge cohorts, and Caliper Life Sciences LabChips for the typhoid Vi vaccine trial.

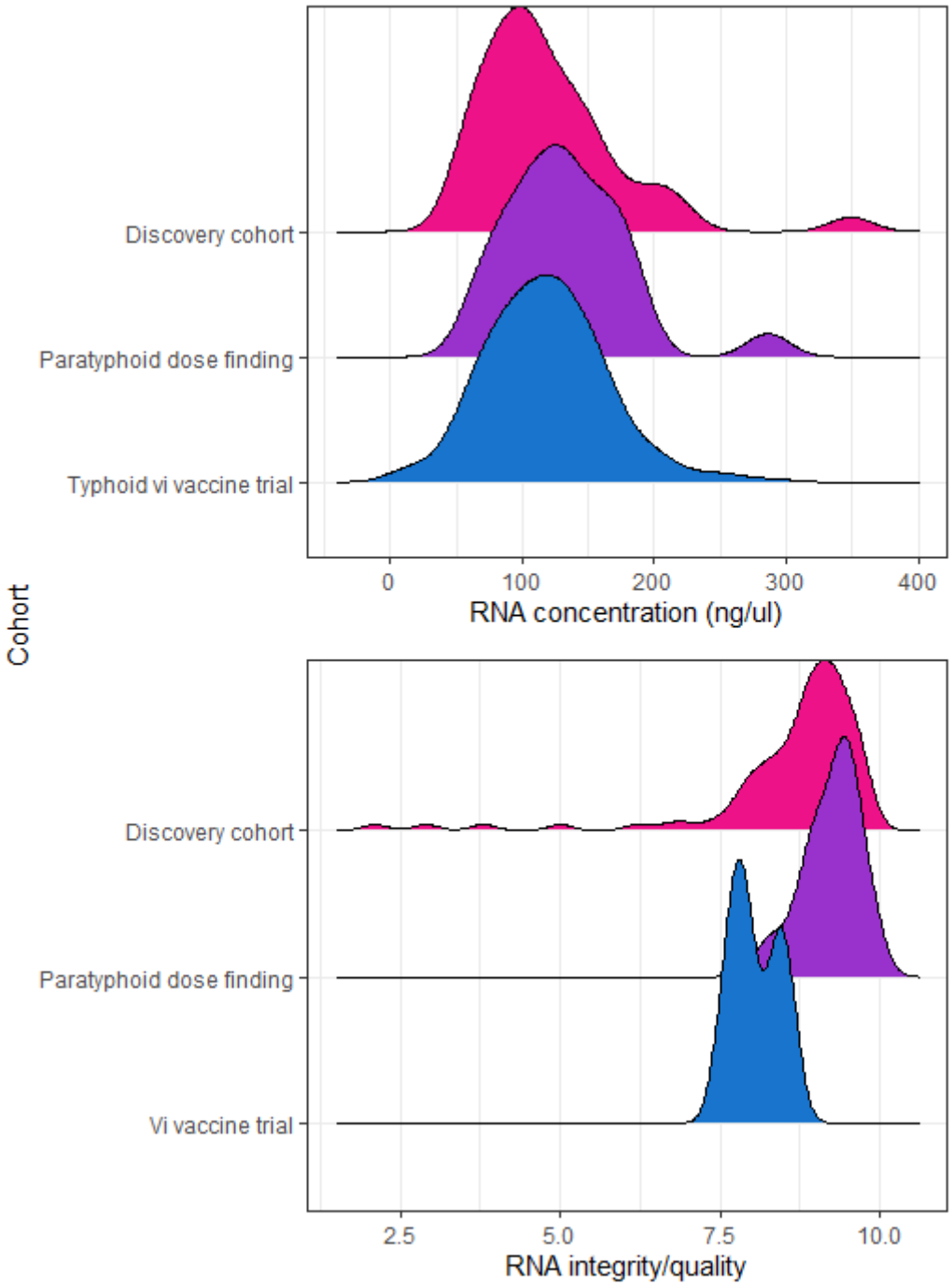


Figure S4.2: Total reads per sample for Paratyphoid dose finding study and Vi vaccine trial RNA samples. Each bar represents one sample, coloured by cohort.

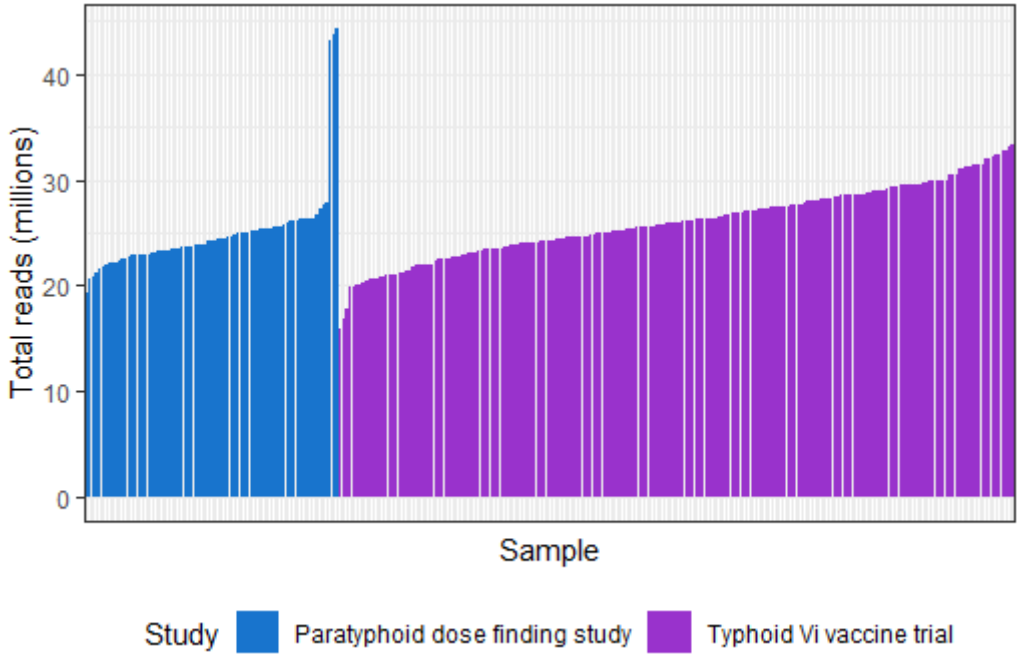
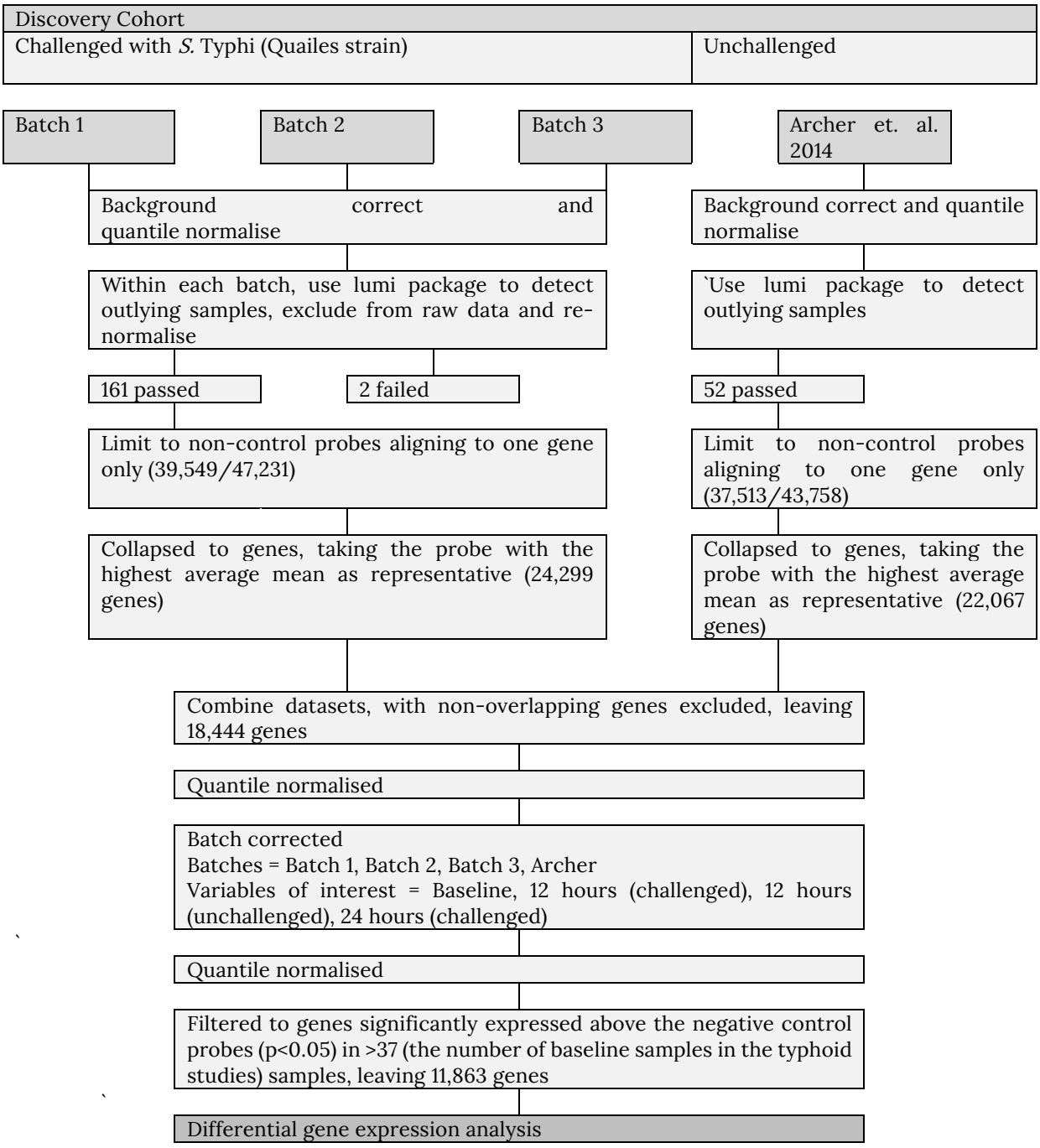


Figure S4.3: Summary of the data processing pipeline



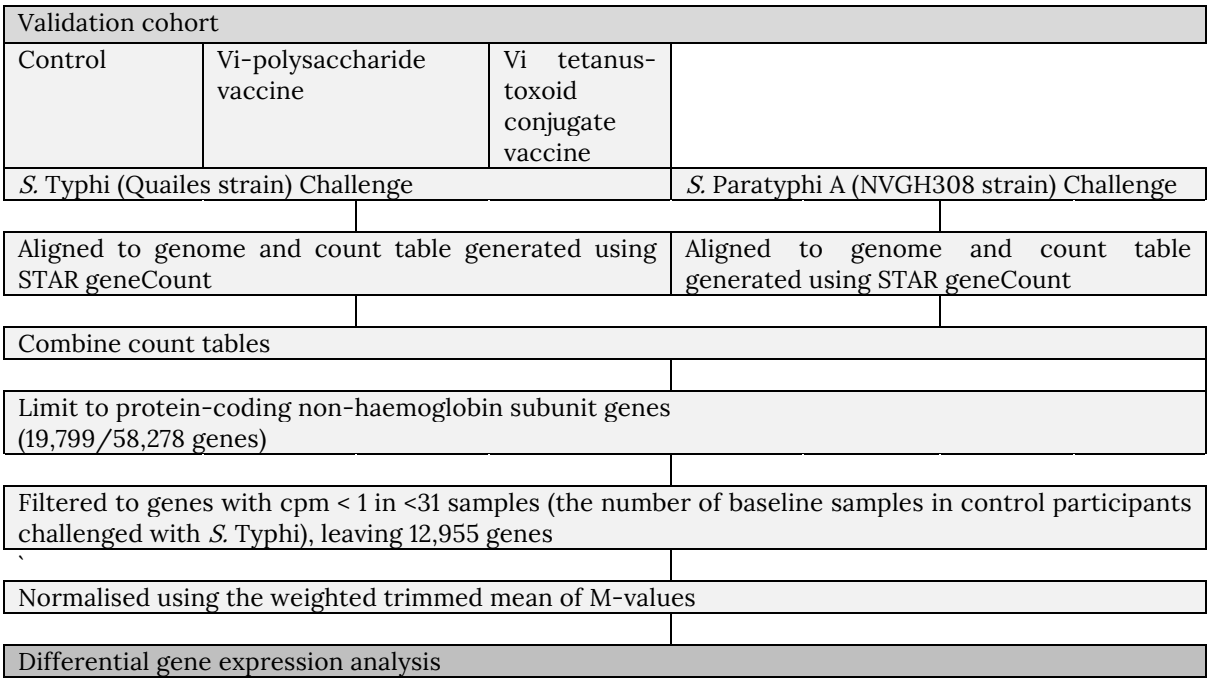
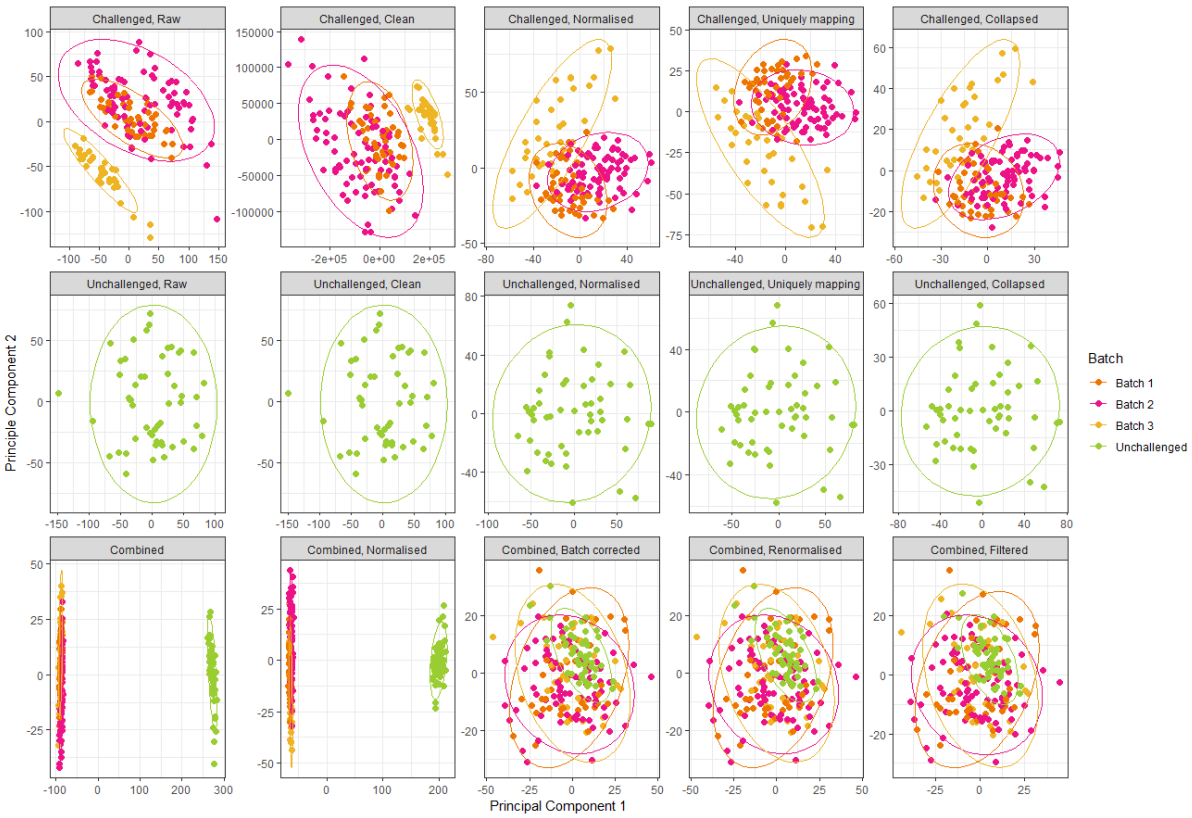
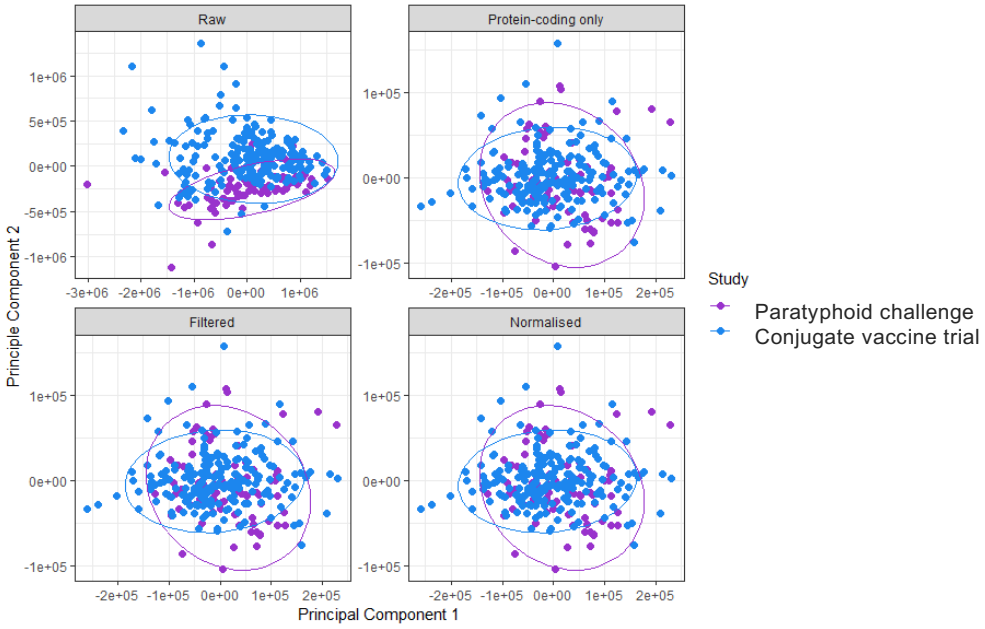

Validation cohort
Control
Vi-polysaccharide vaccine
Vi tetanus-toxoid conjugate vaccine
S. Typhi (Quailes strain) Challenge
S. Paratyphi A (NVGH308 strain) Challenge
Aligned to genome and count table generated using STAR geneCount
Aligned to genome and count table generated using STAR geneCount
Combine count tables
Limit to protein-coding non-haemoglobin subunit genes (19,799/58,278 genes)
Filtered to genes with cpm < 1 in <31 samples (the number of baseline samples in control participants challenged with S. Typhi), leaving 12,955 genes
Normalised using the weighted trimmed mean of M-values
Differential gene expression analysis

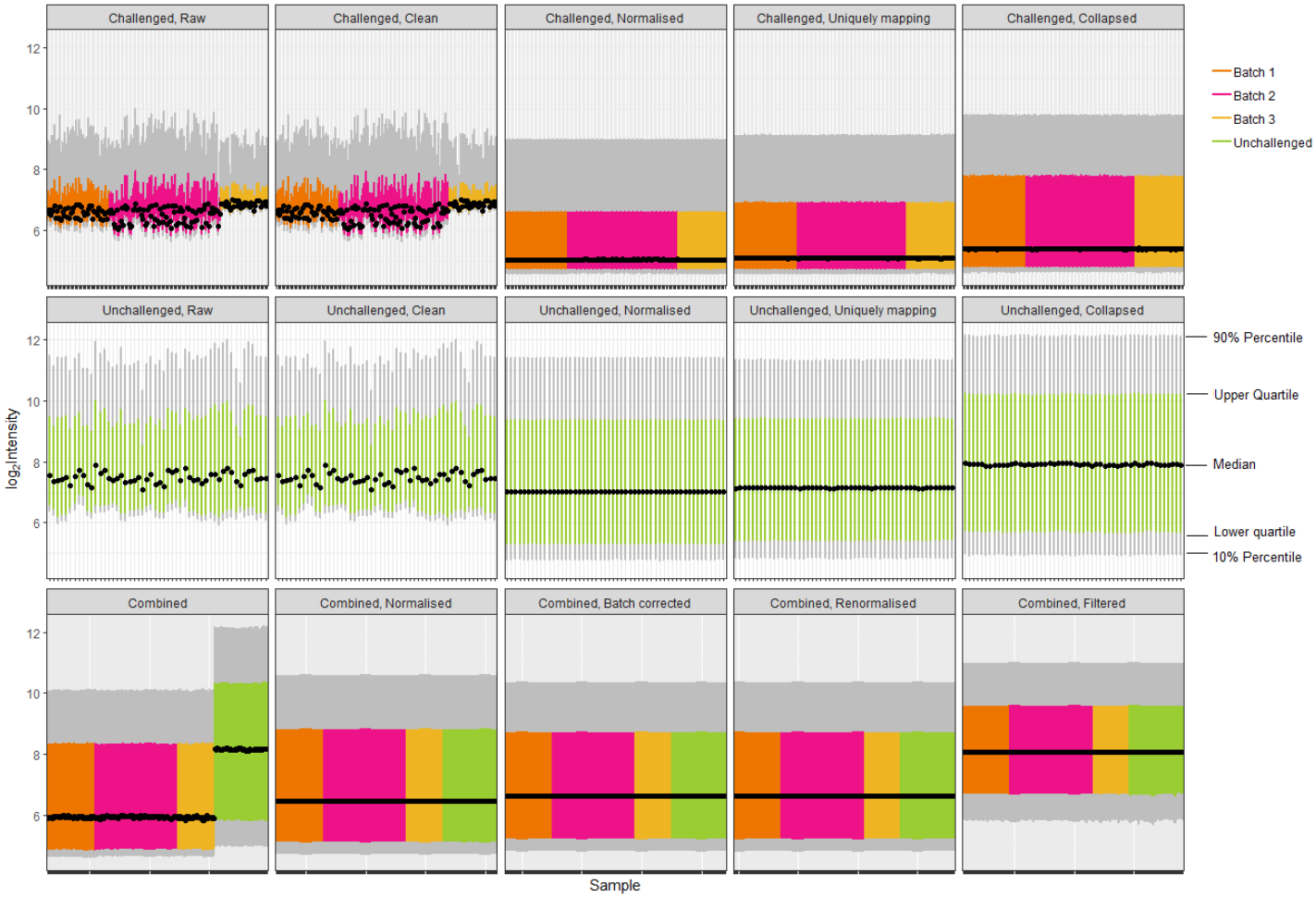
Figure S4.4: (a) the first two principal components of gene expression count tables (b) and distribution of probe intensities at each stage of the data processing pipeline, colour coded by batch
Discovery cohort



Validation cohort



b. Discovery cohort



Validation cohort

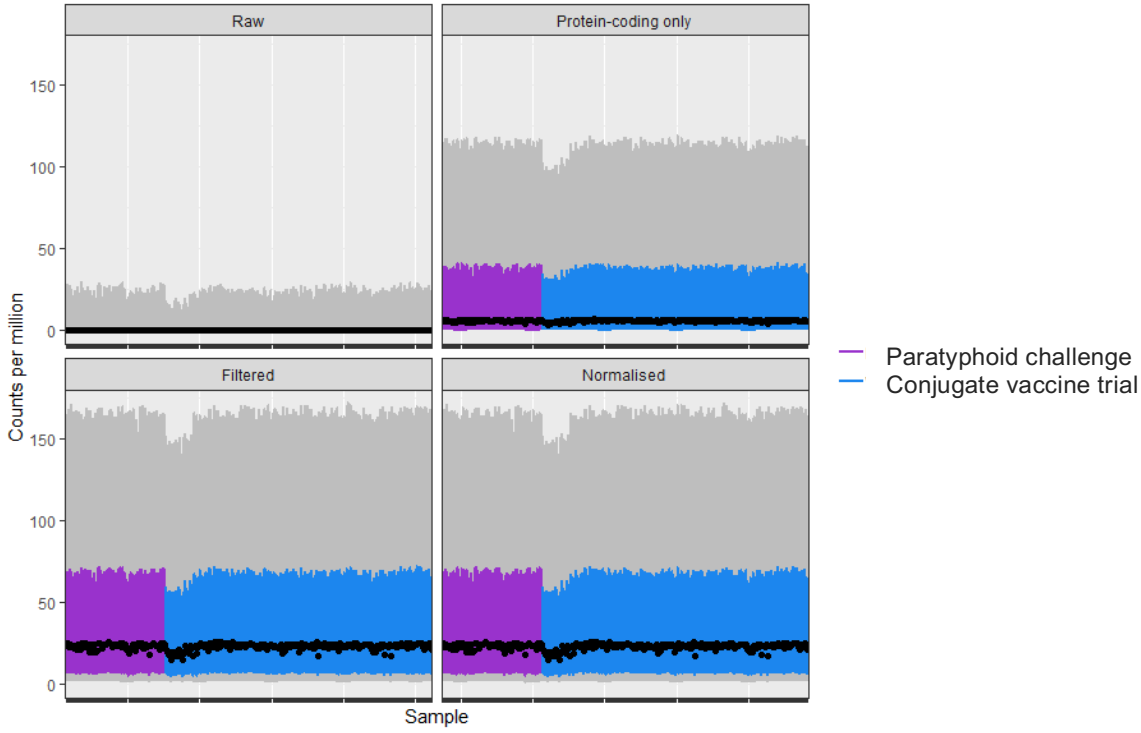


Figure S4.5: Top five most significantly over-represented pathways amongst up- and downregulated genes in each cohort (FDR < 0.05), ranked by p value. P = 0.05 is indicated by a dotted line, with bars crossing the line indicating a pathway with p < 0.05.

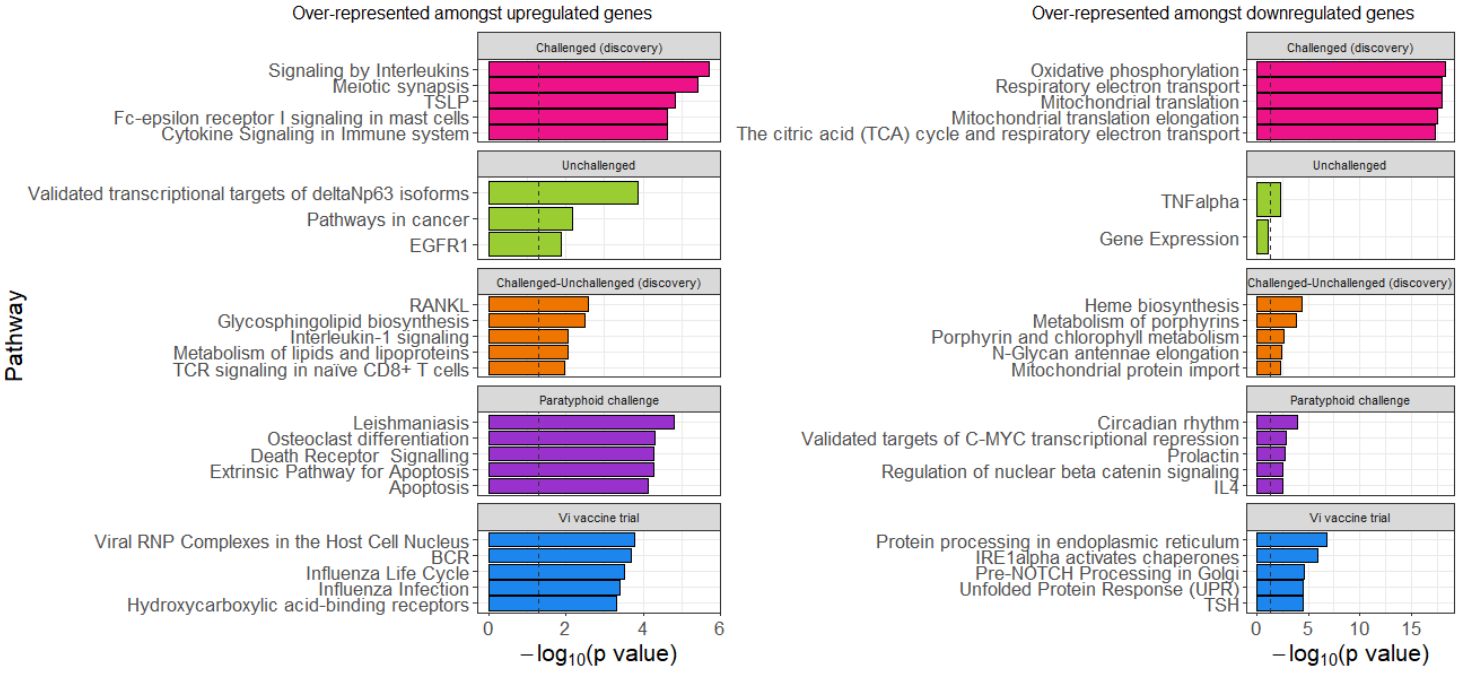


Figure S4.6: Significance (-$\log_{10}$(p value)) of the over-representation of AhR and HIF-1 binding sites amongst up and downregulated genes in different cohorts. P = 0.05 is indicated by a dotted line, with bars crossing the line indicating p < 0.05

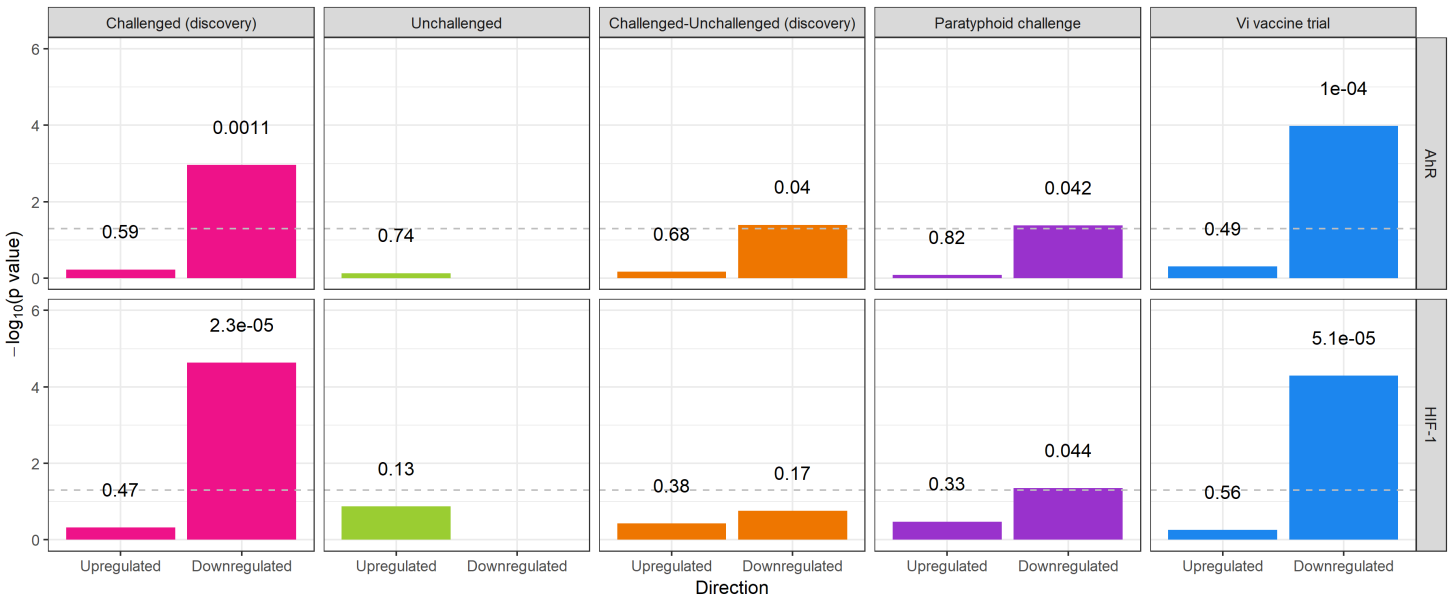


Figure S4.7: Changes in the expression of *AHR* and *HIF1A* 12 hours post-challenge relative to baseline in each cohort. P values are shown for a one-sample t-test with a null hypothesis of a log2(fold change) of zero.

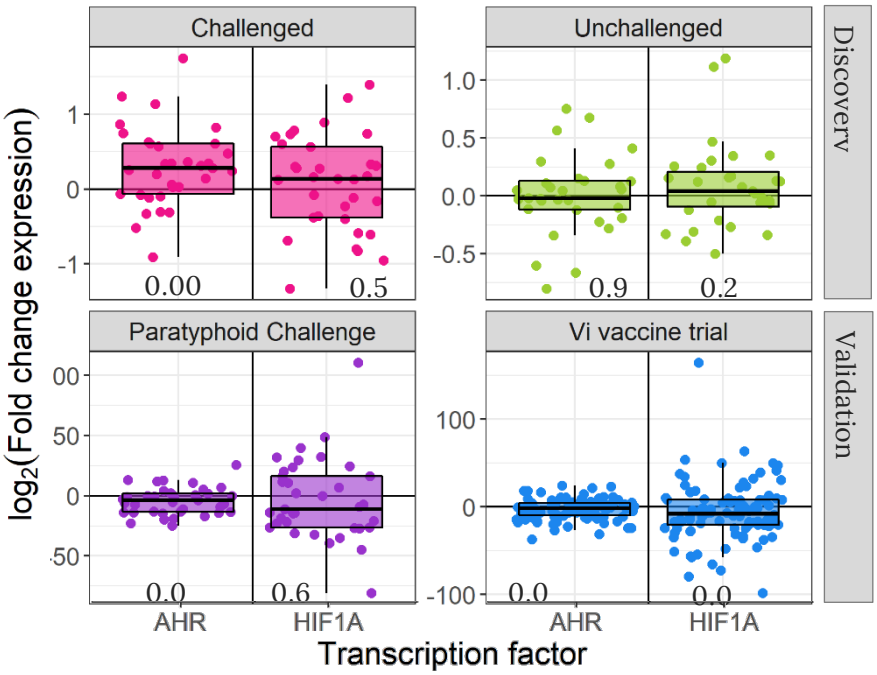


Figure S4.8: Z-score standardised median fluorescence intensity of each neutrophil surface marker (rows) under differing conditions (columns).

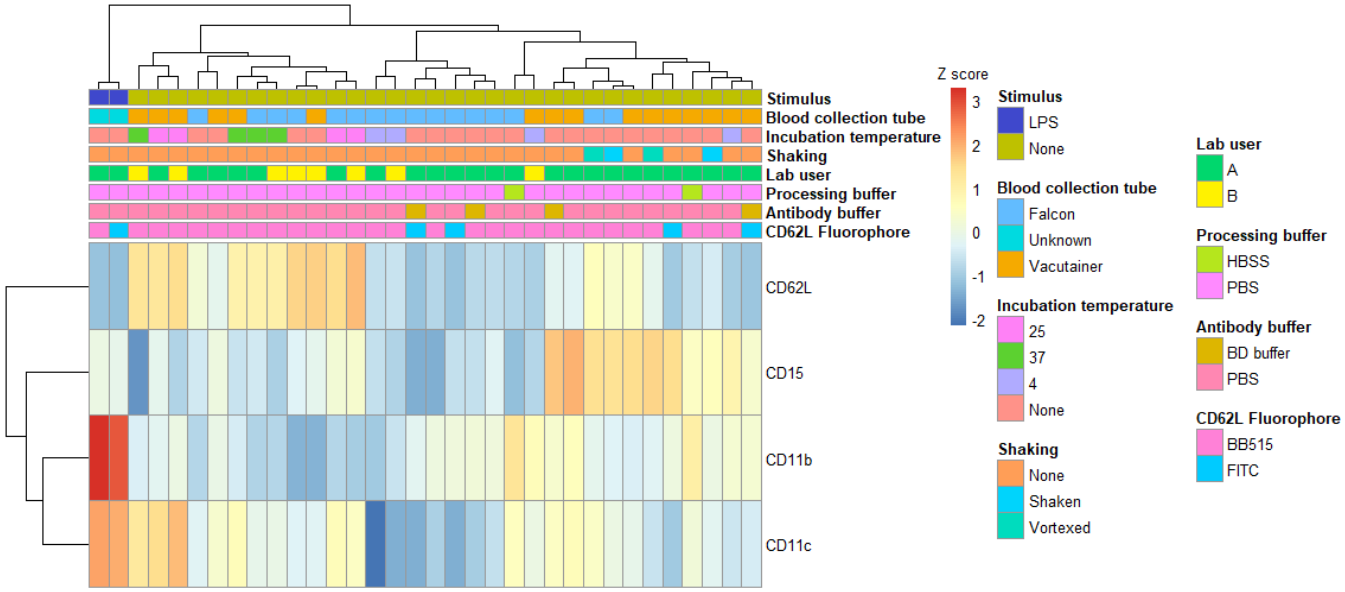


Figure S4.9: Overlap between gene sets over- (NES > 0) or under-represented (NES < 0) in the response of those who did not develop enteric fever compared with those who did.

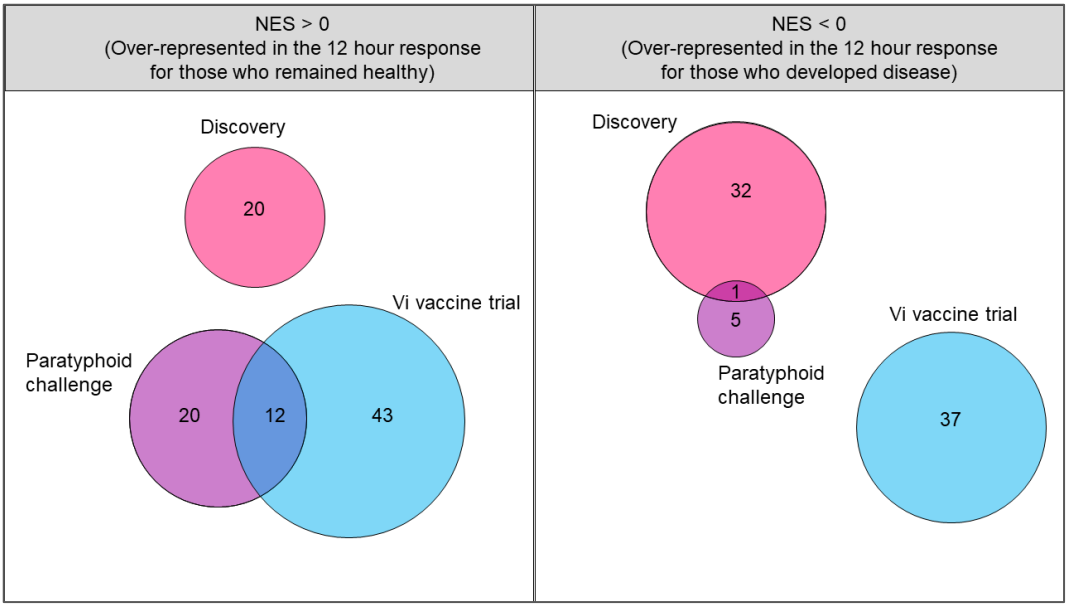


Figure S4.10: Significance ($-\log_{10}$(p value)) of the over-representation of AhR and HIF-1 binding sites amongst up and downregulated genes in stimulated THP-1 and Hep2 cells. P value = 0.05 is indicated by a dotted line, with bars crossing the line indicating $p < 0.05$

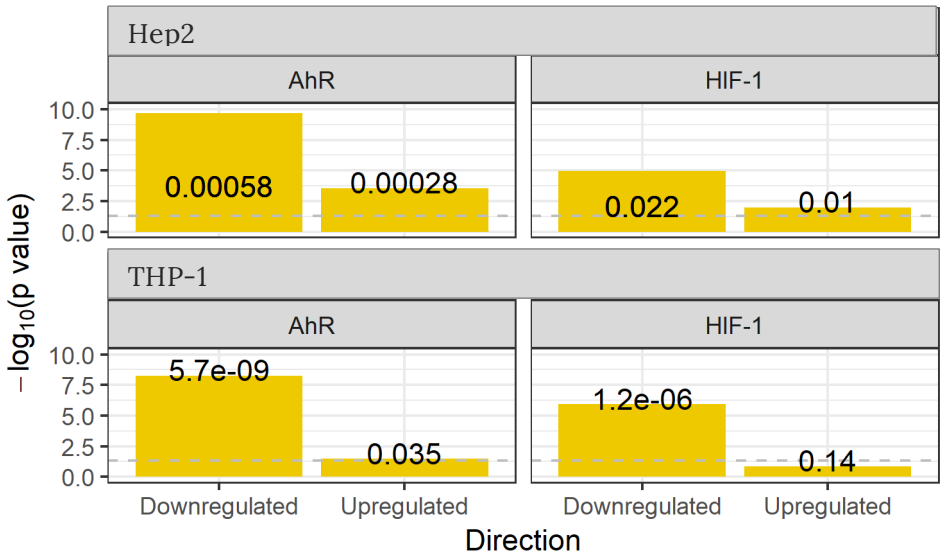


Figure S4.11: Changes in the expression of *AHR* and *HIF1A* one hour post-stimulation relative to baseline in each cell line. P values are shown for a one-sample t-test with a null hypothesis of a $\log_2$(fold change) of zero.

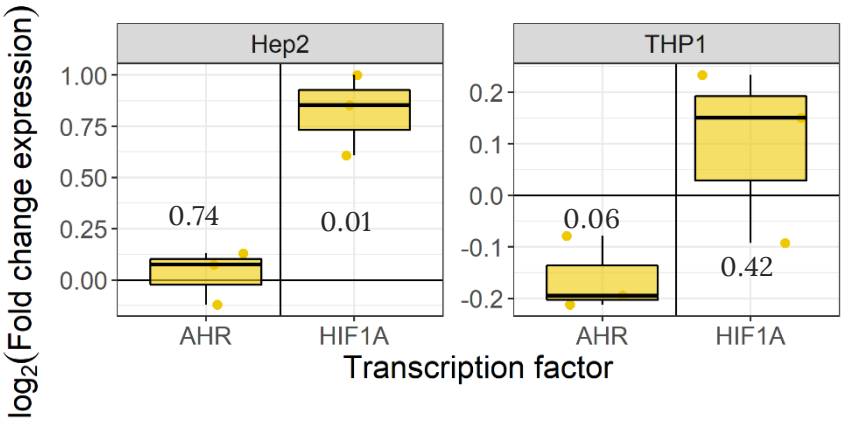


Figure S5.1: Differential expression analysis methods

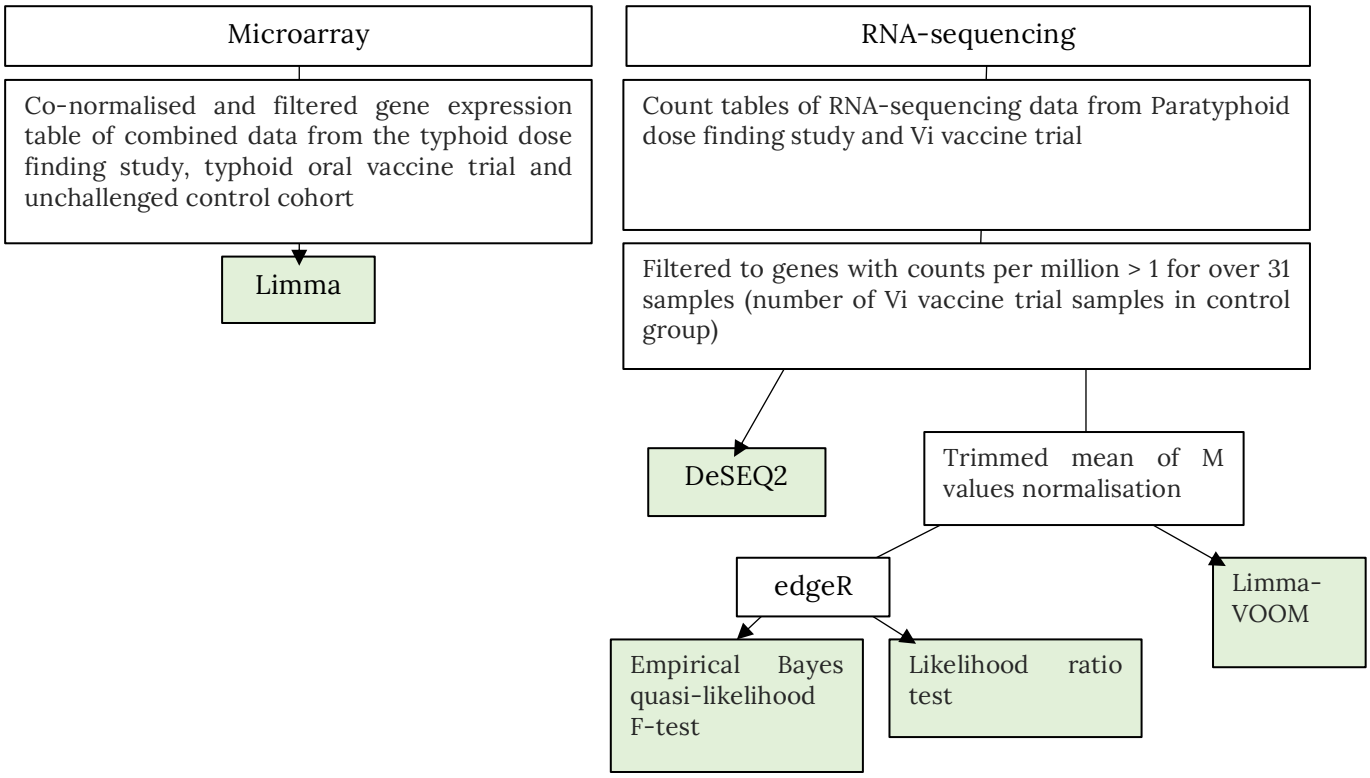


Figure S5.2: Expression levels of genes associated with outcome in at least one subgroup or differential analysis method, in $\log_2$(Intensity) for the first row and $\log_2$(counts per million) for the subsequent rows. Each point corresponds to a participant, with those who went on to develop enteric fever shown in pink and those who remained healthy in blue. The subgroup where each gene was found to be significant is highlighted in dark blue.

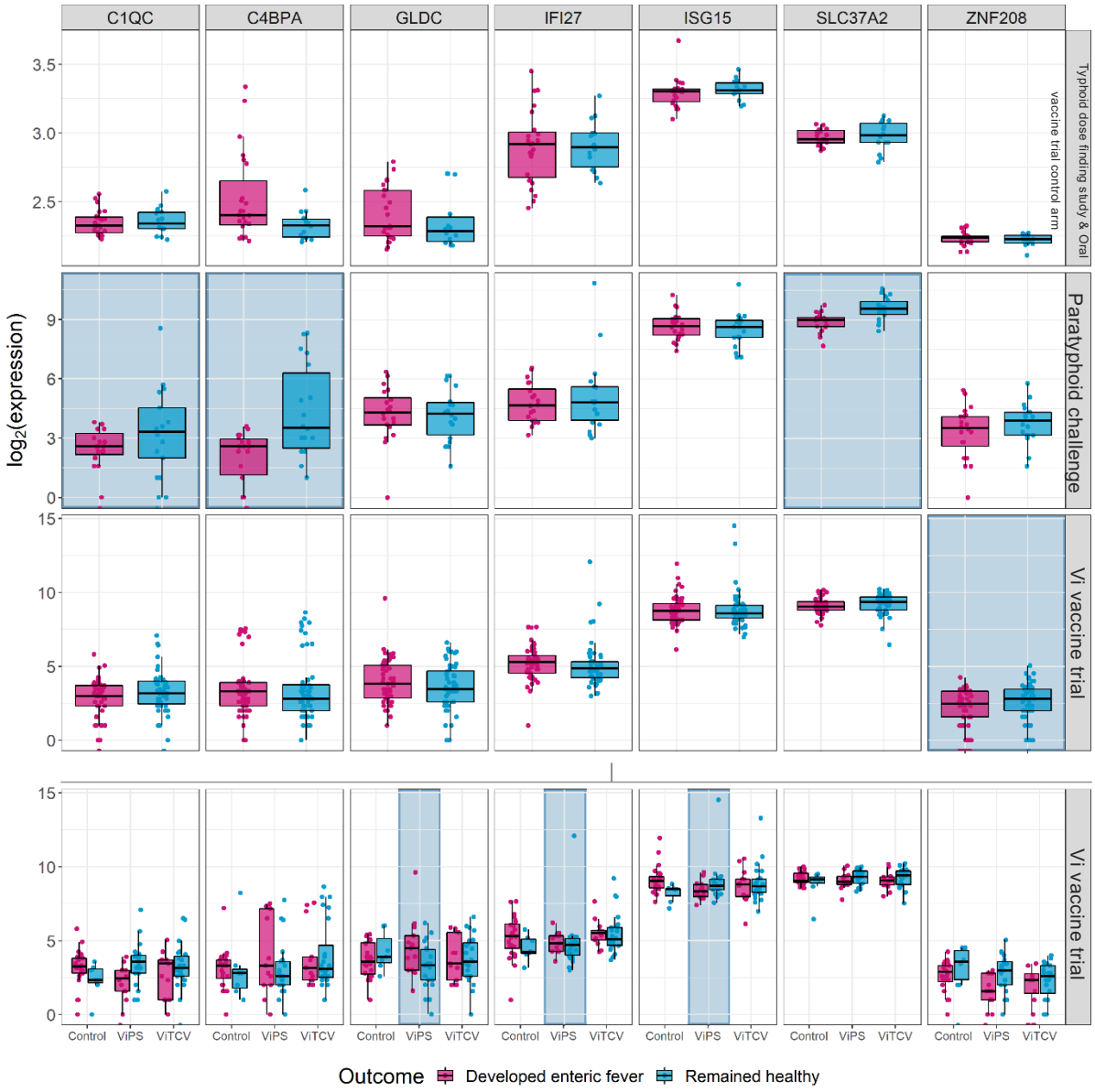


Figure S5.3: Correlation between levels of whole blood *C1QA, C1QB* and *C1QC* mRNA in counts per million, and concentration of plasma C1q. Points are coloured by outcome of challenge. A linear regression line is shown in grey with shaded 95% confidence intervals. The Pearson's product moment correlation coefficient (R) for each correlation is shown, as is a p value for a test of R being zero.

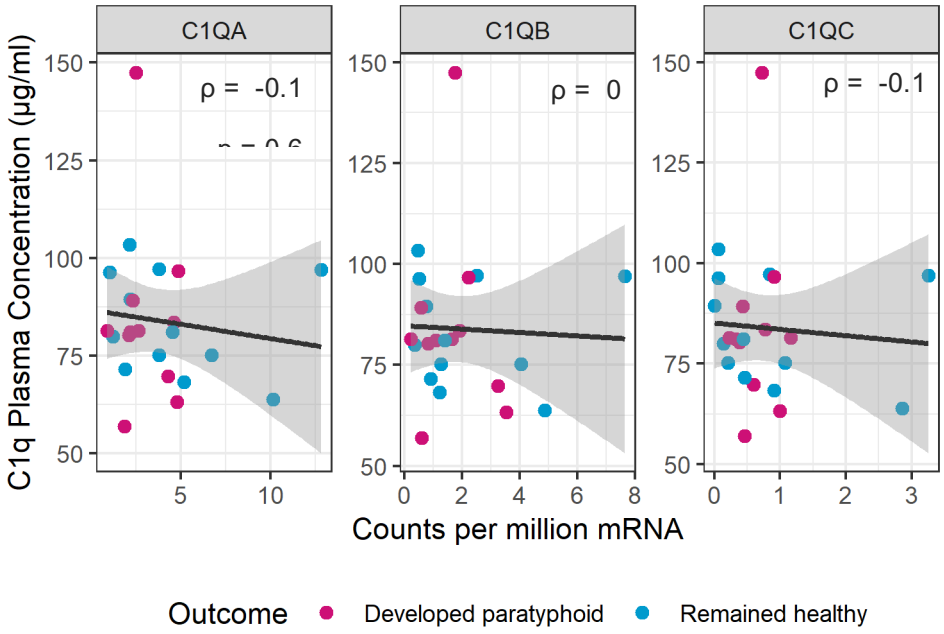


Figure S5.4: Correlation between estimated HLA type dosages (%) derived from RNA-sequencing of samples from two different time points (n=10). Rows display the data from an individual participant with each point corresponding to an HLA type. Correlations are shown for HLA types estimated at low resolution (1 digit), intermediate resolution (2 digits) and high resolution (3 or more digits). The dashed line indicates a slope of 1 with y intercept 0.

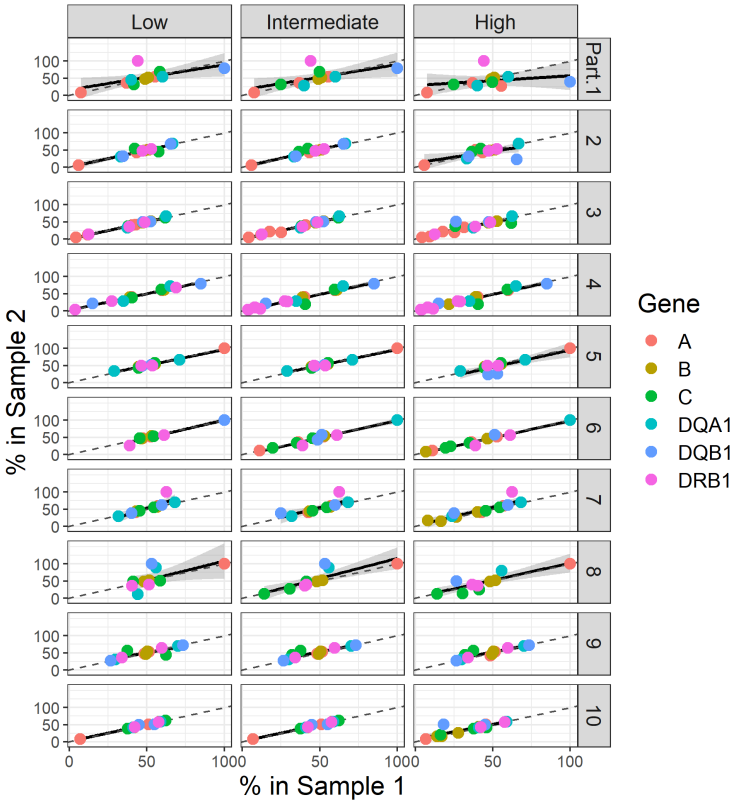


Figure S5.5: a. Regression coefficients with 95% confidence intervals for HLA types associated ($p < 0.05$) with expression of their encoded mRNA, at baseline, 12 hours post-challenge, and with the change in gene expression 12 hours post-challenge relative to baseline. The size of the point reflects the significance of the association.
b. Expression of HLA genes (Z standardised intensity for microarray expression, Z standardised counts per million for RNA-sequencing expression, and the $\log_2$(fold change) in gene expression for change in expression). Participants are grouped by whether they were homozygous negative (dosage < 25%), heterozygous (25% < dosage < 75%) or homozygous positive (dosage > 75%) for each of the HLA types significantly associated with expression.

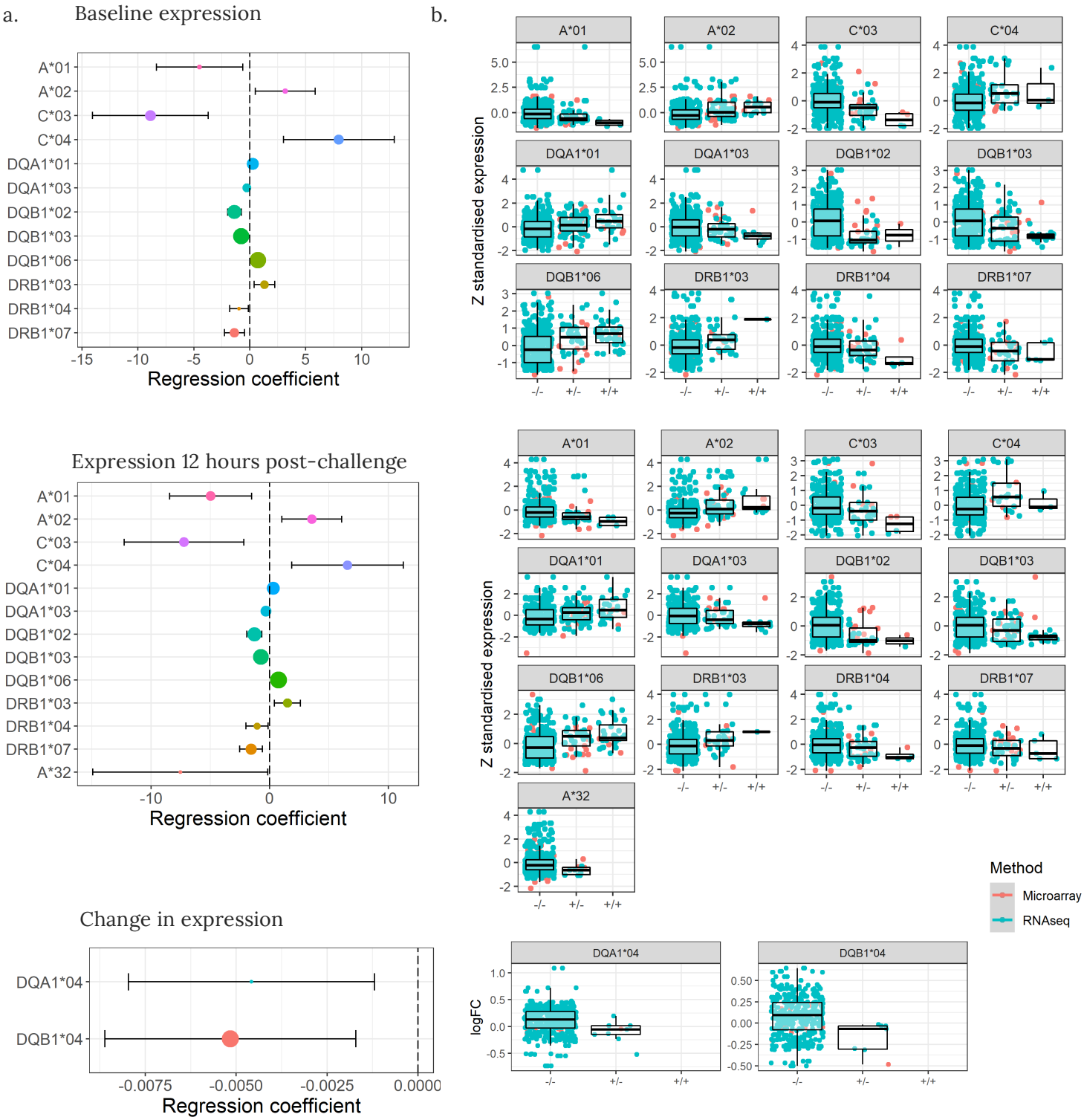


Figure S5.6: Regression coefficients for the relationship between HLA type and HLA expression, compared between the human challenge cohorts and HapMap lymphoblastoid cell lines (LCLs). Each point corresponds to a HLA type, coloured in grey if they weren't significantly associated ($p < 0.05$) with expression in any cohort, blue if they were associated with expression in the LCLs, pink if significant in the human challenge cohort, and purple if significant in both.

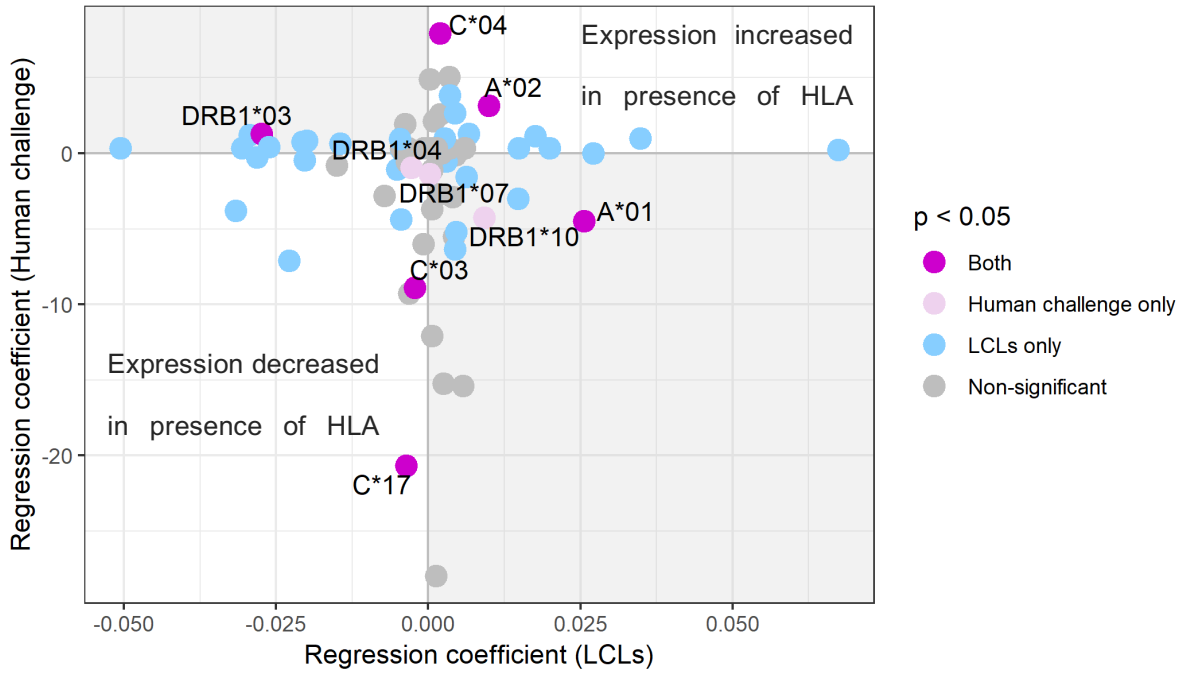


Figure S5.7: Sankey diagram showing the proportion of SNPs and genotyped participants passing quality control for association analysis

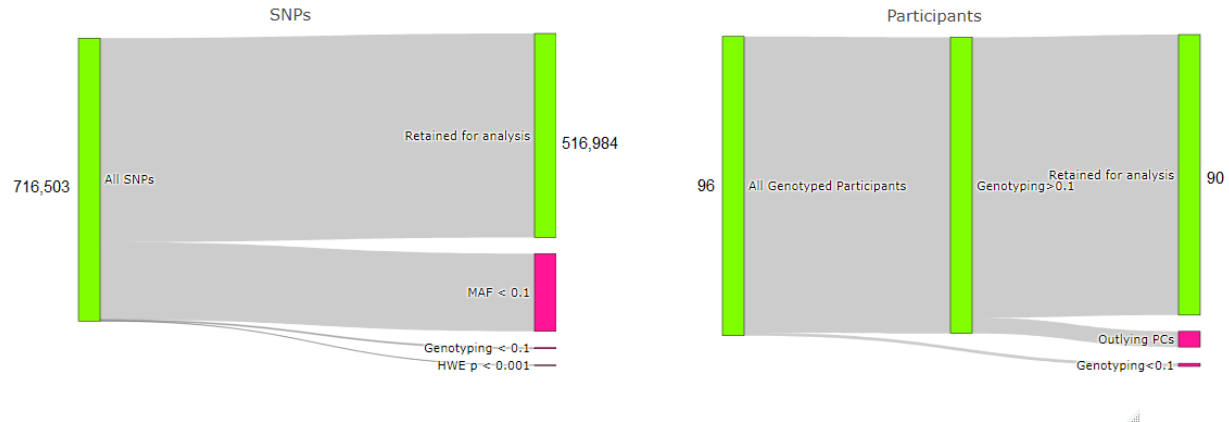


Figure S5.8: The first two principal components for the genotype of each participant, coloured by outcome. The grey dashed box separates points within (retained for association analysis) and outside (excluded) two standard deviations from the mean for principal components 1 and 2.

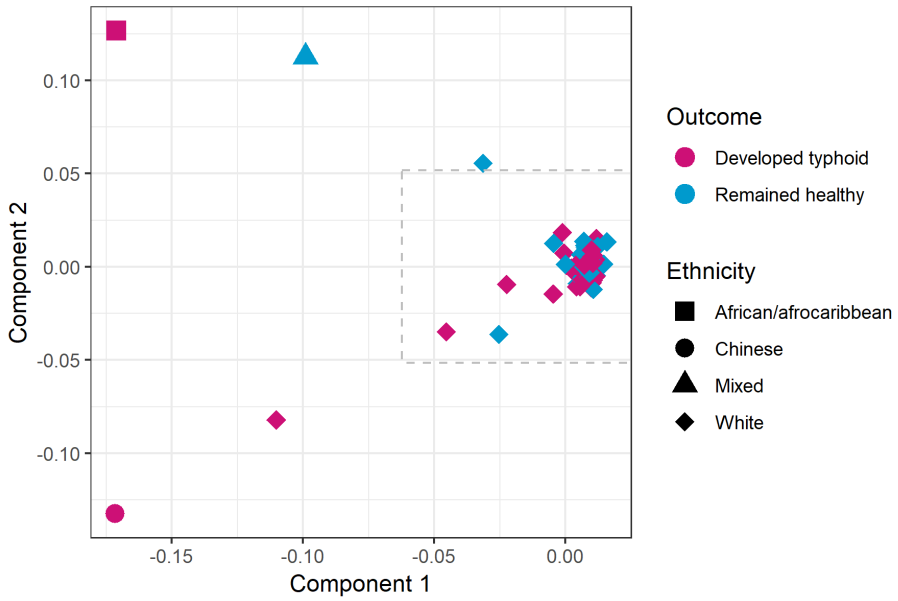


Figure S5.9: Frequencies of different genotypes for the SNPs with $p < 0.0001$ for association with outcome of challenge, ordered by significance.

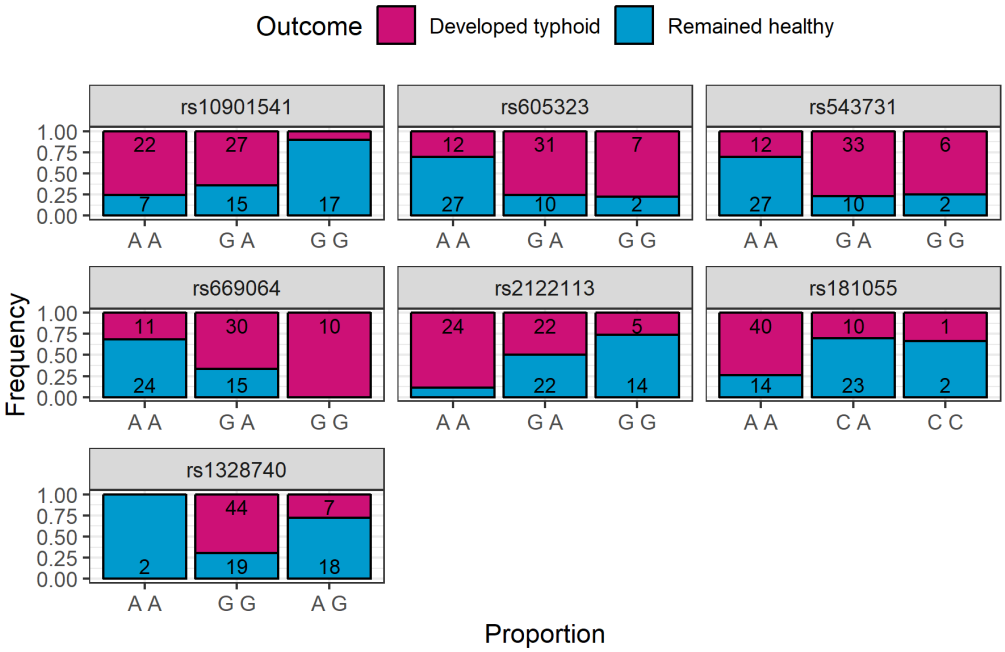
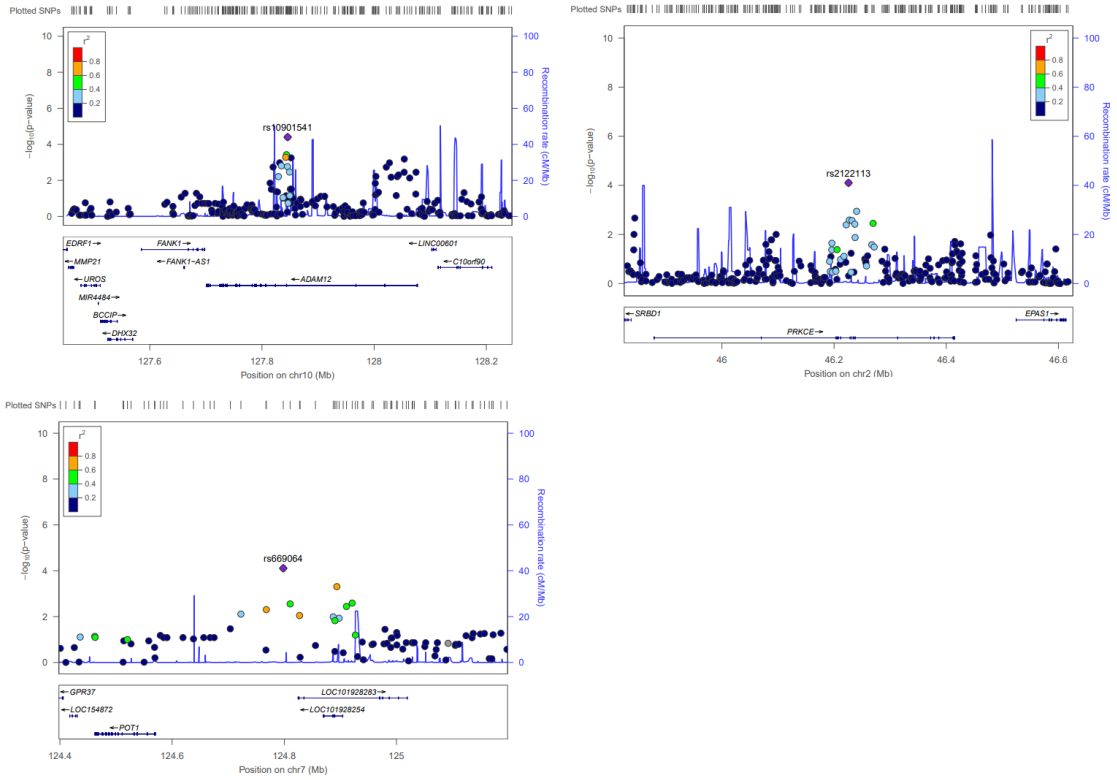


Figure S5.10: Loci of SNPs rs10901541**,** rs2122113 and rs669064 (highlighted in purple), with chromosomal position plotted on the x axis and $-\log_{10}$(p value) and recombination rate on the y axis. The significance of other SNPs in the surrounding loci (+/- 400kB) are also shown. A rug plot above each locus indicates the position of SNPs in this dataset. SNPs are coloured by their linkage disequilibrium (r^2) estimates.



Appendix II: Supplementary tables

Table S3.1: Function of cytokines analysed in this chapter

Name		Gene names	Function
BDNF	Brain-derived neurotrophic factor	BDNF	Promotes the survival and differentiation of selected neuronal populations of the peripheral and central nervous systems
CD40L	CD40 ligand	CD40LG CD40L TNFSF5 TRAP	Co-stimulates T-cell proliferation and cytokine production
EGF	Epidermal growth factor	EGF	Stimulates the growth of various epidermal and epithelial tissues
FASL	Tumour necrosis factor ligand superfamily member 6	FASLG APT1LG1 CD95L FASL TNFSF6	Binds to TNFRSF6/FAS, a receptor that transduces the apoptotic signal into cells
FGFB	Fibroblast growth factor 2	FGF2 FGFB	Regulation of cell survival, cell division, cell differentiation and cell migration
GROA	Growth-regulated alpha protein	CXCL1 GRO GRO1 GROA MGSA SCYB1	Chemotactic activity for neutrophils
HGF	Hepatocyte growth factor	HGF HPTA	Potent mitogen for mature parenchymal hepatocyte cells
ICAM1	Intercellular adhesion molecule 1	ICAM1	During leukocyte trans-endothelial migration, ICAM1 engagement promotes the assembly of endothelial apical cups
IF	Complement factor I	IF CFI	Trypsin-like serine protease that plays an essential role in regulating the immune response by controlling all complement pathways
IFNB	Interferon beta	IFNB1 IFB IFNB	Has antiviral, antibacterial and anticancer activities
IFNG	Interferon gamma	IFNG	In addition to having antiviral activity, has important immune-regulatory functions. It is a potent activator of macrophages
IL10	Interleukin-10	IL10	Major immune regulatory cytokine that acts on many cells of the immune system where it has profound anti-inflammatory functions
IL13	Interleukin-13	IL13 NC30	Inhibits inflammatory cytokine production
IL15	Interleukin-15	IL15	Cytokine that stimulates the proliferation of T-lymphocytes. In neutrophils, stimulates phagocytosis
IL17F	Interleukin-17F	IL17F	Involved in stimulating the production of other cytokines such as IL6, IL8 and CSF2. Also involved in stimulating the proliferation of peripheral blood mononuclear cells and T-cells
IL18	Interleukin-18	IL18 IGIF IL1F4	A pro-inflammatory cytokine primarily involved in polarized T-helper 1 (Th1) cell and natural killer (NK) cell immune responses
IL1A	Interleukin-1 alpha	IL1A IL1F1	Involved in the inflammatory response, being identified as an endogenous pyrogens
IL1B	Interleukin-1 beta	IL1B IL1F2	Potent pro-inflammatory cytokine. Initially discovered as the major endogenous pyrogen,

			induces prostaglandin synthesis, neutrophil influx and activation, T-cell activation and cytokine production, B-cell activation and antibody production
IL1RA	Interleukin-1 receptor antagonist protein	IL1RN IL1F3 IL1RA	Inhibits the activity of interleukin-1 by binding to receptor IL1R1 and preventing its association with the coreceptor IL1RAP for signalling
IL2	Interleukin-2	IL2	Required for T-cell proliferation and other activities crucial to regulation of the immune response
IL21	Interleukin-21	IL21	Cytokine with immune-regulatory activity. May promote the transition between innate and adaptive immunity
IL22	Interleukin-22	IL22 ILTIF ZCYTO18 UNQ3099/PRO10096	Cytokine that contributes to the inflammatory response in vivo
IL31	Interleukin-31	IL31	Activates STAT3 and possibly STAT1 and STAT5
IL4	Interleukin-4	IL4	Participates in B-cell activation
IL5	Interleukin-5	IL5	Induces terminal differentiation of late-developing B-cells to immunoglobulin secreting cells
IL6	Interleukin-6	IL6 IFNB2	A potent inducer of the acute phase response. Plays an essential role in the final differentiation of B-cells into Ig-secreting cells Involved in lymphocyte and monocyte differentiation. Required for the generation of T(H)17 cells
IL7	Interleukin-7	IL7	Stimulating the proliferation of lymphoid progenitors. It is important for proliferation during certain stages of B-cell maturation.
IL8	Interleukin-8	CXCL8 IL8	Chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is also involved in neutrophil activation
IL9	Interleukin-9	IL9	Supports IL2 independent and IL4 independent growth of helper T-cells.
LIF	Leukaemia inhibitory factor	LIF HILDA	Induces terminal differentiation in leukemic cells
MCP1	C-C motif chemokine 2	CCL2 MCP1 SCYA2	Exhibits a chemotactic activity for monocytes and basophils but not neutrophils or eosinophils
MCP3	C-C motif chemokine 7	CCL7 MCP3 SCYA6 SCYA7	Attracts monocytes and eosinophils, but not neutrophils
MIG	C-X-C motif chemokine 9	CXCL9 CMK MIG SCYB9	Cytokine that affects the growth, movement, or activation state of cells that participate in immune and inflammatory response. Chemotactic for activated T-cells. Binds to CXCR3.
NGF	Beta-nerve growth factor	NGF NGFB	Important for the development and maintenance of the sympathetic and sensory nervous systems
PAI1	Plasminogen activator inhibitor 1	SERPINE1 PAI1 PLANH1	Serine protease inhibitor. Inhibits TMPRSS7
SCF	Kit ligand	KITLG MGF SCF	Regulation of cell survival and proliferation, hematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration and function, and in melanogenesis
TGFA	Transforming growth factor alpha	TGFA	Mitogenic polypeptide that is able to bind to the EGF receptor/EGFR and to act synergistically

			with TGF beta to promote anchorage-independent cell proliferation
TGFB	Transforming growth factor beta-1	TGFB1 TGFB	Regulates the growth and differentiation of various cell types and is involved in various processes, such as normal development, immune function, microglia function and responses to neurodegeneration
TNFA	Tumor necrosis factor	TNF TNFA TNFSF2	A potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia, Under certain conditions it can stimulate cell proliferation and induce cell differentiation
TNFB	Lymphotoxin-alpha	LTA TNFB TNFSF1	Lymphotoxin is produced by lymphocytes and is cytotoxic for a wide range of tumour cells in vitro and in vivo
VCAM 1	Vascular cell adhesion protein 1	VCAM1	Appears to function in leukocyte-endothelial cell adhesion
VEGF	Vascular endothelial growth factor A	VEGFA VEGF	Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth
VEGF D	Vascular endothelial growth factor D	VEGFD FIGF	Growth factor active in angiogenesis, lymphangiogenesis and endothelial cell growth

Table S4.1: Overview of samples which underwent transcriptional profiling in the challenged discovery cohort, by participant. Samples from participants who developed typhoid are shown in red and from participants who stayed healthy in blue. Samples which were identified as outliers and excluded from the analysis are marked with an "E".

Study	Typhoid dose escalation					
Batch	1			2		
Participant	0	12	24	0	12	24
1						
2						
3						
4						
5						E
6						
7						
8						
9						
10						
11						
12						
13						
14					E	
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						
25						
26						
27						
28						
29						
30						
31						
32						
33						
34						
35						
36						
37						
38						
39						
40						
41						

Study	Oral vaccine trial		
	3		
Participant	0	12	24
2026			
2029			
2031			
2036			
2039			
2043			
2045			
2046			
2053			
2055			
2059			
2062			
2070			
2072			
2082			
2083			
2091			
2092			
2096			
2097			

Total # samples			
	0	12	24
Batch 1	19	27	0
Batch 2	0	41	41
Batch 3	18	17	0

Table S4.2: Variations in the protocol for isolation and staining of white blood cells for each date of challenge

Experiment	1	2	3	4
Date of challenge	25th April 2017	15th May 2017	31st May 2017	6th July 2017
Number of participants	6	6	3	9
Time points	0h, 12h	0h, 12h, 24h	0h, 12h, 24h	0h, 12h
Positive control	-	Whole blood stimulation with 100ng/ml S. Typhi LPS for 30 min	Whole blood stimulation with 100ng/ml S. Typhi LPS for 30 min	Whole blood stimulation with 100ng/ml S. Typhi LPS for 30 min
Negative control	-	-	3 unchallenged volunteers had blood samples taken and processed alongside challenge participants	-
Temperature	4ºC where possible	4ºC where possible	4ºC where possible	Room temperature
Anti-CD62L fluorescent antibody	1µl FITC-CD62L	4µl BB515-CD62L	4µl BB515-CD62L	4µl BB515-CD62L OR 1µl FITC-CD62L
Consistency in protocol	96 well plates used for 0h and Eppendorf tubes for 12h due to lack of plate spinners in Containment level 3	Inaccurate pipette used for making up antibody mixture	Consistent	Consistent

Table S4.3: Correlation statistics for the log_2fold change 12 hours post-challenge between the challenged discovery cohort and two validation cohorts. Genes were subset to those differentially expressed (FDR<0.05) in the discovery cohort, and either correlated log_2fold change in each validation cohort, or further subset to those differentially expressed in the validation cohort only. The Pearson correlation coefficient (R), p-value for a test of association between the two variables and % genes which are up- or down-regulated in the same direction in both cohorts is shown.

Cohort	Cut-off	Statistic	Unchallenged	Challenged	Challenged-Unchallenged
			Discovery cohort FDR < 0.05		
Paratyphoid dose finding	None	R	0.94	0.65	0.53
		p-value	0.00	0.00	0.00
		Same direction	73%	85%	82%
	p < 0.05	R	0.96	0.87	0.81
		p-value	0.00	0.00	0.00
		Same direction	100%	97%	93%
	FDR<0.05	R	0.96	0.91	0.88
		p-value	0.00	0.00	0.00
		Same direction	100%	99%	100%
Vi vaccine trial	None	R	0.96	0.47	0.20
		p-value	0.00	0.00	0.01
		Same direction	87%	71%	64%
	p < 0.05	R	0.97	0.75	0.52
		p-value	0.00	0.00	0.00
		Same direction	100%	90%	79%
	FDR<0.05	R	0.96	0.85	0.55
		p-value	0.00	0.00	0.34
		Same direction	100%	97%	80%

Table S4.4: Pathways enriched amongst HIF-1 and AhR target genes which were specifically downregulated in response to challenge (FDR < 0.05 in the Challenged-Unchallenged response comparison)

Transcription factor	Pathway Name	Source Database	Adjusted p value
HIF1	Protein processing in endoplasmic reticulum	KEGG	$5x10^{-4}$
	Protein export	KEGG	$8x10^{-4}$
	TSH	NETPATH	0.004
	TNF-alpha	NETPATH	0.02
	Class I MHC mediated antigen processing & presentation	REACTOME	0.02
AHR	ErbB1 downstream signalling	PID NCI	0.02
	Gene Expression	REACTOME	0.02
	Metabolism of amino acids and derivatives	REACTOME	0.02
	ER-Phagosome pathway	REACTOME	0.02
	Antigen processing-Cross presentation	REACTOME	0.02
	Translation	REACTOME	0.03
	Protein processing in endoplasmic reticulum	KEGG	0.03
	RNA transport	KEGG	0.04

Table S5.1: Number of cis- and trans- eQTLs identified at each time point (FDR < 0.05) before and after limiting analysis to SNPs previously associated with gene expression in neutrophils, B cells or monocytes

	Pre-filtering		Post-filtering	
	No. cis-eQTLs	No. trans-eQTLs	No. cis-eQTLs	No. trans-eQTLs
Baseline	1,491	339	1,279	347
12 hours	417	6	610	11
Acute disease	1,046	201	970	184
12 hours - Baseline	0	0	5	0
Acute disease - Baseline	11	0	29	0

Table S5.2: Number of a. cis-eQTLs and b. trans-eQTLs (FDR < 0.05) for immune related genes (InnateDB Innate Immunity Genes) at each time point. Genes are ordered by the p value of the most significant eQTL

Number of cis-eQTLs			
Gene	Baseline	12 hours post-challenge	Acute disease
LGALS2	2	2	2
TUFM	2	0	2
AMFR	5	5	5
STAT6	5	1	4
LPCAT2	1	1	1
VASP	1	0	1
IRF5	5	2	5
BTN3A2	5	5	2
LST1	1	1	1
LY86	0	0	2
ANXA4	7	0	7
SF3A1	1	1	1
SERPINB9	4	0	4
TRIM66	0	0	5
UCP2	1	0	1
IREB2	6	1	6
AP3B1	1	0	1
SPI1	1	2	0
CTSK	1	0	0
INSIG1	0	0	1
GNAI3	4	3	0
CTSS	3	0	3
NR1H3	4	0	0

CARD16	1	0	0
SLC11A1	3	1	0
HLA-E	1	1	0
DR1	0	0	2
SIVA1	0	1	0
CLEC1B	1	0	0
PPP4C	1	0	0
CFB	0	0	3
TECPR1	2	1	0
PPP3R1	0	0	1
MYH9	2	0	0
CTSB	1	0	0
ERAP1	0	0	3
CAMKK2	0	1	1
CARD9	1	0	0
MKNK1	1	0	0
C1QBP	0	1	0
MAPK9	2	0	0
PSMB8	0	0	0
KPNA1	0	2	0
ITGB2	0	1	0

No. of trans-eQTLs		
Gene	Baseline	Acute disease
IRF5	8	6
LGALS2	0	1
TUFM	16	0
AMFR	2	0
PYHIN1	1	0