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Genomics of immune response to typhoid and cholera vaccines

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Considerable variation in antibody response (AR) was observed among recipients of an injectable typhoid vaccine and an oral cholera vaccine. We sought to find whether polymorphisms in genes of the immune system, both innate and adaptive, were associated with the observed variation in response. For both vaccines, we were able to discover and validate several polymorphisms that were significantly associated with immune response. For the typhoid vaccines, these polymorphisms were on genes that belonged to pathways of polysaccharide recognition, signal transduction, inhibition of T-cell proliferation, pro-inflammatory signalling and eventual production of antimicrobial peptides. For the cholera vaccine, the pathways included epithelial barrier integrity, intestinal homeostasis and leucocyte recruitment. Even though traditional wisdom indicates that both vaccines should act as T-cell-independent antigens, our findings reveal that the vaccines induce AR using different pathways.

1. Introduction

The impact of vaccines on the control and eradication of many infectious diseases cannot be overstated. Yet, vaccines do not confer equal protection to all vaccine recipients (table 1). A recent dramatic example is that among the 1521 cases of a mumps outbreak in a 2009 summer camp in New York, only 9.1% had not previously received a mumps vaccination [1]. Because vaccines primarily work through the immune system of the recipient, it is important to quantify variability in immune response among vaccinees and to understand the causes of variability. As a matter of fact, a new branch of omics—called vaccinomics—has evolved that aims to understand the mechanisms of heterogeneity in immune responses to vaccines [2]. Many twin and family studies [3–6] have shown that vaccine immune response is highly heritable. Vaccinomics, therefore, posits that heterogeneity in host genetic markers results in variations in vaccine-induced immune responses and aims to identify these factors for predicting and minimizing vaccine failures or adverse events. While there are multiple parameters by which to measure immune response in a vaccinee [7–9], levels of various immunoglobulins have traditionally been used. This is because immune response usually involves the production of antibodies or immunoglobulins by B lymphocytes. It is now well established that for various vaccines, genetic effects play a dominant role in immune response [10–12]. Epigenomic effects are also becoming evident [13]. Quantification of immune response at various stages after vaccination is also important, because the level of early response may predict later response, which is known to be important is determining the extent of protection offered by the vaccine. In a study on a yellow fever vaccine [14], gene expression signatures in the blood a few days after vaccination—early innate response-related molecular signature—have been found that could predict later antibody titres with about 90% accuracy. Furthermore, these investigators also identified that a calcium/calmodulin-dependent protein kinase type IV largely controls antigen-specific antibody production, thus providing insights into the mechanism of immune response to the vaccine. With respect to a hepatitis B vaccine [15], an extended HLA signature has been found to be associated with non-response, indicating that individuals who possess this signature may require multiple doses or vaccines containing increased amounts of antigen. Studies on immune response to vaccines and on other aspects of vaccine research have spawned

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Table 1. Impact of vaccines in the twentieth and twenty-first centuries. Data source: http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/appendices/G/impact-of-vaccines.pdf.

disease	% decrease in annual morbidity	disease	% decrease in annual morbidity
smallpox[a]	100	*Haemophilus influenzae*[a] (<5 years of age)	99
diphtheria[a]	100	hepatitis A[b]	91
pertussis[a]	89	hepatitis B[b] (acute)	83
tetanus[a]	99	pneumococcus[b] (invasive)	
polio[a] (paralytic)	100	all ages	30
measles[a]	>99	<5 years of age	74
mumps[a]	98	rotavirus[b] (hospitalizations <5 years of age)	88
rubella[a]	>99	varicella[b]	89
CRS[a]	100		

[a]Based on a comparison of twentieth century annual morbidity and current morbidity.
[b]Based on a comparison of pre-vaccine era estimated annual morbidity and current morbidity.

the development of an online network called vaccine investigation and online information network [16]. Vaccine immunogenicity is determined in part not only by the chemical and physical nature of microbial antigens and adjuvants, but also by the genetic make-up of vaccine recipients [17]. The impact of genetic variation in vaccinees on AR has also been obtained in some detail [18] for some vaccines, but not for vaccines against enteric diseases such as typhoid and cholera.

2. The typhoid vaccine study

Primary failure, as assessed by post-vaccination antibody levels, occurs in 23–40% of recipients of typhoid vaccine [19,20]. The available typhoid vaccine is a polysaccharide (PS) vaccine, an inactivated subunit vaccine composed of long chains of sugar molecules that make up the surface capsule of *Salmonella enterica* serotype *typhi* (*Salmonella typhi*). The essential feature of immune response to a pure PS vaccine is that it is typically T-cell-independent. A PS vaccine stimulates B cells and non-cognate T cells without the assistance of cognate T cells [21,22]. Because data on the role of genetic variation in vaccinees on AR to PS vaccines are scanty, we undertook a study using an available typhoid vaccine. The objective of this study was to quantify the extent of variation in AR to a widely used Vi PS vaccine for typhoid and to discover associations of polymorphisms in candidate genes of vaccinees with AR.

(a) Methods of vaccinee recruitment, biospecimen collection, assays and statistical analyses

Potential vaccinees were recruited with written informed consent, provided that (i) she/he had not had fever lasting for more than three consecutive days in the past 1 year, and (ii) she was not pregnant or lactating. Individuals ($n = 1000$), unrelated at least to the first-cousin level, aged 12 years or older, inhabiting a socioeconomically depressed locality of Kolkata (formerly Calcutta), India, were thus recruited.

Each individual recruited into the study was provided, by intramuscular injection (marketed in India as Typherix® by GlaxoSmithKline), 0.5 ml vaccine containing 25 µg of the cell surface Vi PS extracted from *S. typhi* Ty2 strain with the excipients sodium chloride, sodium dihydrogen phosphate dihydrate, disodium phosphate dihydrate, phenol and water. From each study participant, a blood sample was collected immediately prior to vaccination and 28 days post-vaccination. Serum and DNA were isolated from these samples, using standard protocols. The use of Vi PS as a coating antigen in enzyme-linked immunosorbent assay (ELISA) to measure vaccine-induced anti-Vi ARs has been reported to be problematic. The use of PSs (lipopolysaccharide (LPS), *Haemophilus influenzae* type b capsular PS, Vi PS) as coating antigens for immunoassays is plagued by problems such as a poor binding of PSs to ELISA plates and inconsistent results [23 and references therein]. We therefore developed and standardized the Bio-Plex assay that provides much more robust and consistent results than ELISA [24]. The bead assay was performed using the Bio-Plex (Bio-Rad, Hercules, CA) platform. The Vi PS antigen (supplied by Fina Biosolutions, Rockville, MD) was conjugated to Bio-Rad beads (COOH (028)) at a concentration 8.33×10^6 beads ml^{-1} in a total volume of 1.50 ml. Aliquots (50 µl) of human serum were prepared at various dilutions in human serum diluent (PBS, 1% w/v, 5% v/v/ goat serum, 0.05% Tween 20 v/v and 0.1% Kathon v/v). Day-0 serum was diluted 1 : 50, 1 : 400 and 1 : 3200; day-28 serum was diluted 1 : 200, 1 : 1600 and 1 : 12 800 in human serum diluent before incubation with the Vi beads for 3 h. After washing the beads, the bound human IgG was detected by goat anti-human IgG-RPE (1 : 5000 in human serum diluent) by incubation for 30 min. Following the final wash steps, the beads were analysed using the Bio-Plex instrument. A pooled serum with a high anti-Vi IgG response was used to create a standard curve that was assigned an arbitrary value of 200 ELISA units (EU) per ml. The responses in the individual subjects were compared with the standard curve to calculate anti-Vi IgG EU ml^{-1} in the day-0 and day-28 serum.

Single nucleotide polymorphisms (SNPs) in and around 283 autosomal genes from immunological pathways were chosen, using a statistical protocol that took into account differences in interpopulation variation of linkage disequilibrium and allele frequencies, from the HapMap database (http://www.hapmap.org). Genotyping was done using Sequenom (55.7% of assayed SNPs) and Illumina (remaining 44.3% of SNPs) platforms.

AR to vaccination, measured as the difference between 28-day post-vaccination and pre-vaccination antibody levels

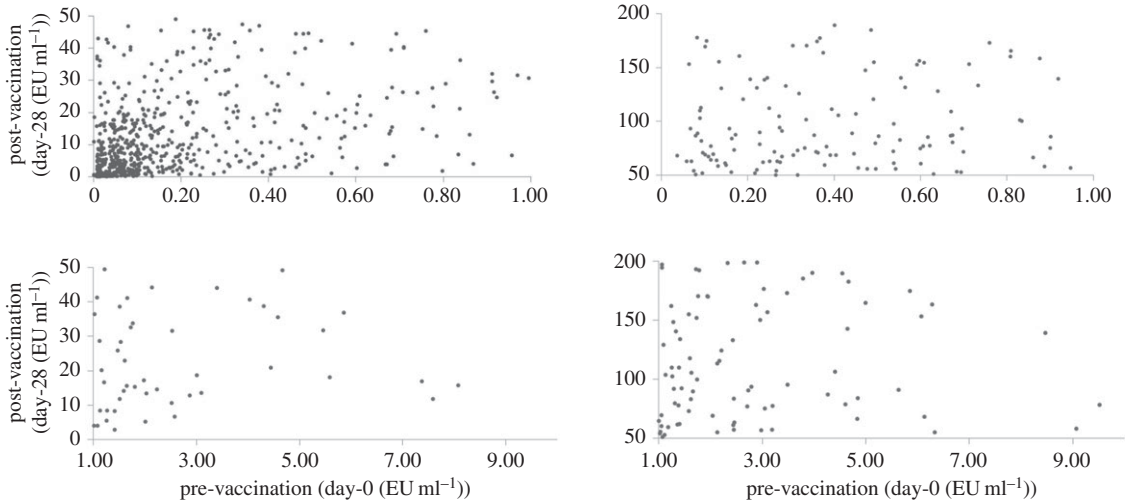

Figure 1. Scatter diagram depicting variation in pre- and post-vaccination Vi-polysaccharide antibody titres among typhoid vaccine recipients. (The enormity of the extent of variation necessitated splitting the single scatter diagram into four quadrants for display.)

(EU ml^{-1}), was $\log_{10}$-transformed (logAR) to induce normality to the frequency distribution. The genotype data were curated by removing (i) loci with less than 90% call rate (8.9% of loci), (ii) non-polymorphic loci with minor allele frequency (MAF) < 0.05 (24.7% of loci with call rate $>$ 90%), (iii) significantly deviant from Hardy–Weinberg equilibrium (4.2% of polymorphic loci), (iv) individuals with less than 75% of loci with valid genotype calls, (v) individuals on whom AR could not be properly assayed (8 of 1000 individuals) and (vi) individuals showing cryptic relationships (more than 80% of loci with identical genotypes: six pairs of individuals satisfied this criterion; one individual from each pair was randomly chosen for final analysis). After data curation, the final dataset comprised genotypes of 984 individuals at 2040 SNPs in 283 genes. Analysis of variance (ANOVA) was performed to test equality of mean values of logAR among subgroups of vaccinees.

For association analysis, we sorted the AR of the vaccinees and grouped the vaccinees into five pentile (20 percentile) groups. We then computed the correlation coefficient between the MAF at the locus and mean AR of vaccinees belonging to the pentile groups, and tested the significance of each correlation coefficient non-parametrically using a resampling technique. Genotypes of individual vaccinees at a locus were randomly permuted, and the correlation coefficient was computed in this permuted sample. This procedure was repeated 1000 times to produce a histogram of correlation coefficients under the null hypothesis $r = 0$. This histogram was then used to compute the p-value associated with the observed r at this locus. After generating the p-values for all loci, the false discovery rate procedure [25] was used to adjust p-values for multiple testing and to identify statistically significant values of r at the 5% level of significance. All significant allelic associations with logAR detected using the above-described procedures were also validated using genotype data using PLINK v. 1.06 [26] (http://pngu.mgh.harvard.edu/~purcell/plink/). We have used the quantitative trait association module of PLINK, and have used empirical significance levels. We have also tested the equality of mean values of the response among individuals stratified by genotype at each locus using PLINK.

Because association studies are known to result in many false-positive inferences, we generated an internal cross-validation sample by randomly splitting the 984 vaccinees into two half-samples of approximately 500 vaccinees each (with approx. 100 vaccinees in each of the five pentile groups based on AR within each half-sample). The first half-sample was used for association discovery and the second half-sample was used for validation of discovered associations. Haplotypes were inferred using PHASE [27] (http://www.stat.washington.edu/stephens/phase.html). Haplotype association analysis was performed using the above-described permutation algorithm for single SNP markers.

(b) Enormous variation in antibody titres among vaccine recipients

The scatter diagram of Vi-PS pre- (day 0) and post- (day 28) vaccination antibody titres among the 984 vaccinees are depicted in figure 1. The extent of variation was enormous. The correlation between pre- and post-vaccination antibody levels was low (0.188) but statistically significant ($p < 0.0005$). The contributions of variation in age, gender or religious backgrounds to the variation in difference between post- and pre-vaccination antibody titres were statistically non-significant ($p > 0.05$).

(c) Genomic association discovery and validation

No statistically significant genetic stratification or substructuring among the vaccines could be detected [28]. The demographic characteristics of the vaccinees in the discovery and validation half-samples were similar and statistically non-significant. In the discovery half-sample, 54 SNPs in and around 43 genes showed significant correlation (p-values were estimated by data permutation) at the 5% level (using multiple-testing correction by FDR method) with AR. The discovered associations were cross-validated using the data on the validation half-sample. Of these 54 SNPs, significant association was found only for eight SNPs—all intronic—in seven genes (table 2). These genes were saturated with additional SNPs, genotype data were generated for the additional SNPs and haplotype associations with AR were calculated; the correlations were all statistically significant [28].

Table 2. Details of the eight SNPs in seven genes that showed significant cross-validated association with antibody response.

gene	chromosome no.	rs no.	nt position	SNP location within gene (mRNA transcript no. in NCBI database)	r^a in discovery half-sample ($n = 492$)	r in validation half-sample ($n = 492$)	p-value using PLINK in the total sample ($n = 984$)
IL1RL1	2	10208293	102332742	Intron 10 (NM_016232.4)	0.95	0.91	0.002
CTLA4	2	231779	204442732	Intron 1 (NM_005214.3)	−0.99	−0.93	0.028
CD86	3	17203439	123278196	Intron 1 (NM_175862.3)	0.95	0.97	0.016
TLR1	4	5743572	38481786	Intron 2 (NM_003263.3)	0.98	0.93	0.05
DEFB1	8	2978873	6717373	Intron 1 (NM_005218.3)	0.98	0.98	0.016
		2977772	6720606	Intron 1 (NM_005218.3)	0.91	0.94	0.013
MAPK8	10	10857564	49282578	Intron 1 (NM_002750.2)	−0.95	−0.96	0.03
IL17D	13	1888001	20183906	Intron 2 (NM_138284.1)	0.94	0.91	0.018

[a]r is the correlation between minor allele frequency and antibody response in EU ml^{-1}.

3. The cholera vaccine study

Cholera is caused by toxicogenic strains of the Gram-negative bacterium *Vibrio cholerae*. Ingestion of contaminated food or water causes cholera. Infected individuals suffer from moderate-to-severe watery diarrhoea that leads to dehydration. Cholera can be fatal if left untreated. Lack of safe water is the major cause of endemicity of cholera in many countries. In recent times, there have been two major outbreaks: in Zimbabwe in 2009 and in Haiti in 2010. Several thousand people died of cholera. These outbreaks prompted the World Health Organization to encourage administration of oral cholera vaccine. *V. cholerae*, has two major serogroups—O1 (serotypes Inaba and Ogawa) and O139—that are responsible for most cholera cases. Members of the serogroups differ in the LPS structures of the outer membrane. Each dose of the oral cholera vaccine contains: formalin-killed *V. cholerae* Inaba, El Tor biotype (strain Phil 6973; 600 EU), heat-killed *V. cholerae* Ogawa classical biotype (Cairo 50; 300 EU), formalin-killed *V. cholerae* Ogawa classical biotype (Cairo 50; 300 EU), heat-killed *V. cholerae* Inaba, classical biotype (Cairo 48; 300 EU) and formalin-killed *V. cholerae* O139 (4260B; 600 EU). Two doses are orally administered 14 days apart.

(a) Methods

The methodology of recruitment of 1000 individuals into the cholera vaccine study was similar to that of the typhoid vaccine study. Serum anti-*V. cholerae* (Inaba 569) LPS—that is, total anti-LPS antibody—was assayed using the Bio-Plex (Bio-Rad) platform. Anti-LPS Ig EU ml^{-1} was estimated using methods similar to those described for the typhoid vaccine study. A vibriocidal assay was performed for each individual with *V. cholerae* O1 Inaba (OS-418), Ogawa (MAK757) and uncapsulated O139 (MO-10T4) strains using sera collected during pre- and post-vaccine trials. Commercially available guinea pig serum (Rockland, Gilbertsville, PA) was used as a source of complement. The sera (100 ml) were added to 100 ml of PBS in the first well to give twofold dilution and subsequent dilutions were made reciprocally up to 4800. Reference rabbit antisera against *V. cholera* O1 Inaba, Ogawa and O139 were included in each set of assay as controls. In every batch of assay, serum obtained from a healthy volunteer and a high-titre antiserum obtained from one of the volunteers in this study were included as negative and positive controls, respectively.

Table 3. Mean and s.d. of serum anti-*V. cholerae* LPS antibody in 948 cholera vaccine recipients pre- and 28 days post-vaccination, and fold change.

	mean	s.d.
pre-vaccination (EU ml^{-1})	22.54	37.95
post-vaccination (EU ml^{-1})	76.89	114.88
fold change (pre/post)	5.42	12.41

(b) Genomic association discovery and validation

After data curation, data on 948 vaccine recipients were available for statistical analyses. Although the vaccine is administered two times, 14 days apart, we considered the antibody level 28 days after the first administration of the vaccine as the post-vaccination response. Similar to the typhoid study, there was considerable variation in immune response to the cholera vaccine (table 3) as reflected by the large standard deviations of pre-vaccination antibody level, as also of 28-days post-vaccination level and fold-change (defined as the ratio of post-vaccination to pre-vaccination antibody levels). The effects of age, gender and religion on variation in fold-change were all statistically non-significant ($p > 0.05$). We tested for existence of cryptic relatedness and population stratification using Eigenstrat [29]; no evidence was detected as all individuals formed a single cluster in the scatterplot of the first three principal components [30] (figure 1).

In the discovery subset of vaccinees, statistically significant correlations with AR, as measured by fold-change, were detected for 159 SNP loci on 93 genes. These correlations were then validated in the validation subset. Most discovered correlations could not be validated, except seven SNPs that are located in and around five genes (rs17180481 and rs17180600 in the flanking region and intron, respectively, of *MARCO* (2q14.2); rs598493 in an intron of *TNFAIP3* (6q23.3); rs3012694 and rs266087 in introns of *RFX3* (9p24.2); rs266087 in an intron of *CXCL12* (10q11.21) and rs6567272 in an intron of *TNFRSF11A* (18q22.1)). Mean AR for vaccinees belonging to the three genotypes for each of these seven SNP loci differed

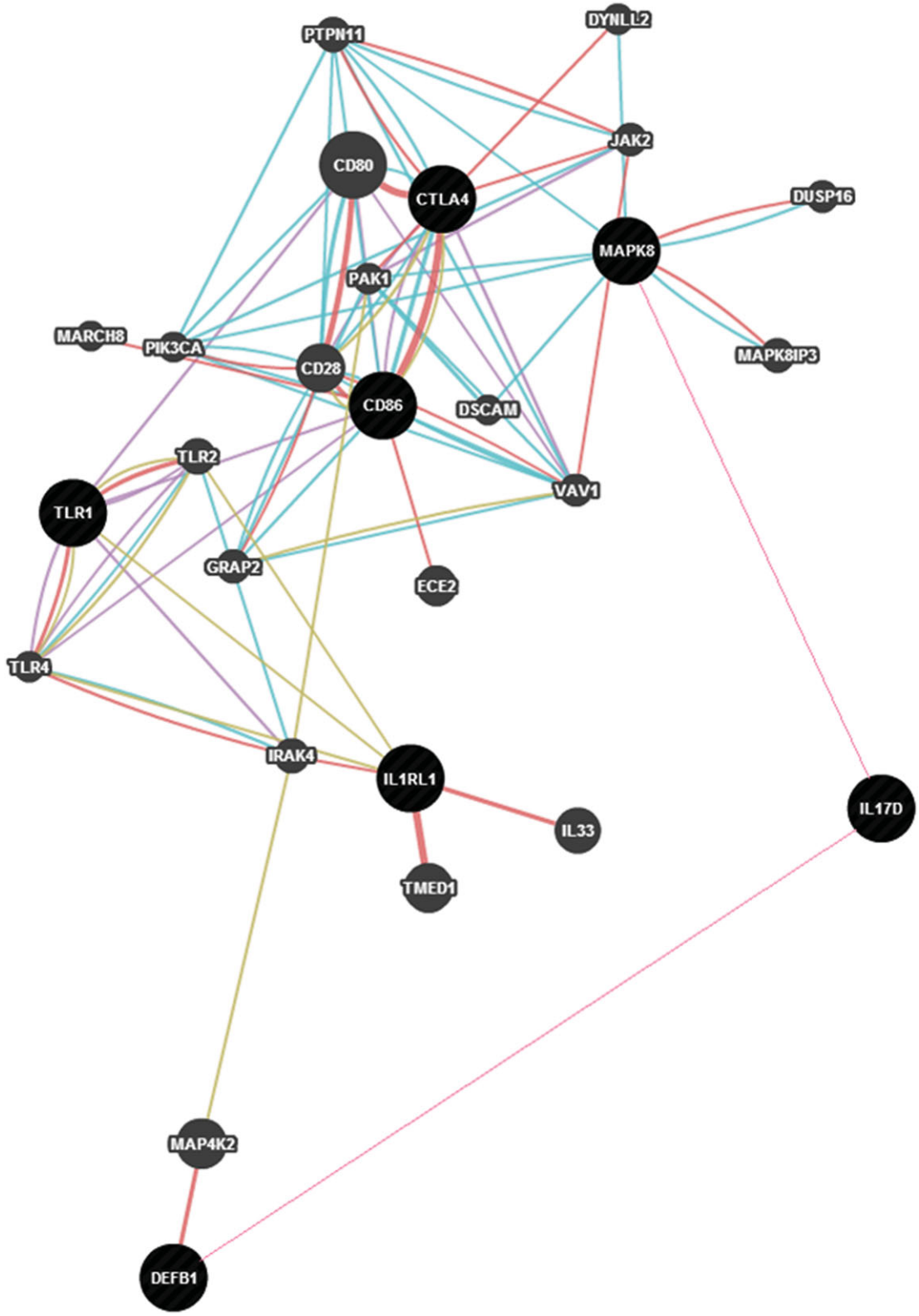

Figure 2. Network of genes associated with antibody response to the typhoid vaccine constructed using GeneMania and depicts physical interaction (red), co-expression (purple), physical interaction (turquoise) and shared protein domains (grey) among the genes. (Online version in colour.)

significantly for only the four SNPs in and around *MARCO*, *TNFAIP3* and *CXCL12* (rs17180481: $p = 0.023$, effect size $= 1.8 \pm 0.3$; rs17180600: $p = 0.026$, effect size $= 1.6 \pm 0.3$; rs598493: $p = 0.001$, effect size $= 3.9 \pm 0.8$; rs266087: $p = 0.014$, effect size $= 2.4 \pm 0.6$), but not for the remaining three.

4. Discussion

Many non-protein antigens, such as PSs and lipids, are not processed and presented along with MHC proteins and hence are not recognized by helper T cells. Therefore, these stimulate antibody production in the absence of helper T cells and are T-independent antigens. Being composed of multiple identical epitopes, most T-independent antigens are polyvalent. Polyvalent antigens can induce cross-linking of surface Ig molecules on B cells, leading to activation of B cells without the requirement of helper T cells.

SNPs in seven genes—*CD80*, *CTLA4*, *TLR1*, *IL1RL1*, *MAPK8*, *IL17D* and *DEFB1*—were found to be associated with variation in AR to the typhoid vaccine. The network

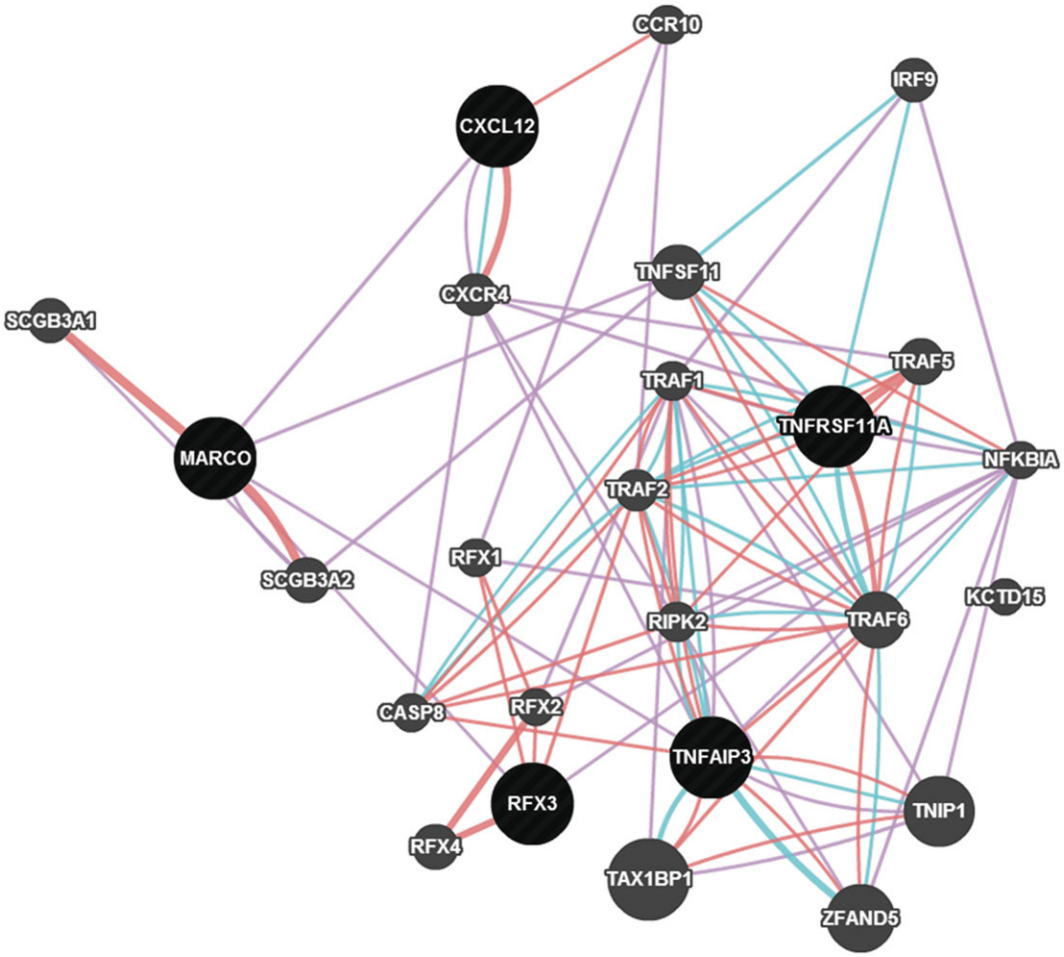

Figure 3. Network of genes associated with antibody response to the cholera vaccine. (Online version in colour.)

relationship among these genes based on their co-expression, co-localization, etc. is presented in figure 2. It has been noted [28] that the B7 family of co-stimulatory molecules, CD86 along with CD80, present on the antigen-presenting cells, interact with CD28/CTLA4 receptors on T cells to provide major non-cognate co-stimulatory signals [31]. The non-cognate T-cell dependence is indicated by the finding of SNPs in *CD86* and *CTLA4* being associated with AR to the PS vaccine. A critical quantitative role for CTLA-4 in governing T- and B-cell homeostasis has also been recently discovered [32]. *TLR1* showed association with the host response to the vaccine. An important immune evasion mechanism in some bacterial pathogens is by downregulation of TLR-mediated immune responses through diminished TLR-mediated cell signalling or expression [33,34]. *TLR1* coexpresses with *CD86* (figure 2). All Toll-like receptors have a Toll-interleukin 1 domain that is responsible for signal transduction. *IL1RL1* participates in this signalling. We have found SNPs in this gene to be significantly associated with AR induced by the Vi-PS vaccine. MAP kinases are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. Therefore, the association of an SNP in *MAPK8* with AR is not unexpected. The involvement of a MAP kinase and *IL17D*, which stimulates the production of other cytokines, can result in pro-inflammatory signals that can induce upregulation of *DEFB1* [35]. We have found multiple SNPs in *DEFB1* to be significantly associated with Vi-PS vaccine response. Thus, the overall picture that emerges from our study is that polymorphisms in genes involved in PS recognition, signal transduction, inhibition of T-cell proliferation, pro-inflammatory signalling and eventual production of antimicrobial peptides are associated with AR to the Vi-PS vaccine for typhoid.

The cholera vaccine is an LPS vaccine. This vaccine, like the Vi-PS typhoid vaccine, is expected to induce antibody production without support of T-helper cells. However, we have found no commonality among the genes that modulate AR to the typhoid and cholera vaccines. One reason for this may be that the routes of administration of these vaccines are different.

Three genes—*MARCO*, *TNFAIP3* and *CXCL12*—were found to have significant roles in modulating AR to the oral cholera vaccine. A single cell layer of the mucosal epithelium essentially regulates intestinal homeostasis [30]. This physical barrier is a prominent part of the innate immune system with damage-healing and infection-limiting properties. *CXCL12* is a homeostatic or constitutive chemokine. The product of this gene plays a key role in damage-healing by stimulating the activation of laminin-specific integrins [36]. *V. cholerae* infection produces strong inflammatory manifestations [37]. CXCL12 is a neutrophil and lymphocyte chemoattractant. It is upregulated in response to *V. cholerae* infection, which results in increased recruitment of polymorphonuclear leucocytes to the site of infection [38]. The LPS in the cholera vaccine possibly provides signals that mimic those of the live bacterium, without actually causing disease. MARCO is a type II transmembrane protein of the class A scavenger-receptor family [39]. The major component of the cholera vaccine is LPS which is a very potent activator of innate immune responses. Surface expression of MARCO is upregulated in a dose- and

time-dependent manner by LPS stimulation [40]. MARCO is upregulated in bacterial infections in macrophages of most tissues; variants in this gene have crucial roles in bacterial binding [41]. *MARCO* and *CXCL12* are co-expressed (figure 3). Epithelial barrier integrity is a possible requirement of adequate response to the whole-cell-killed cholera vaccine. Tight junctions between intestinal epithelial cells mediate the permeability of the intestinal barrier. *TNFAIP3* has a role in promoting intestinal epithelial barrier integrity and in enabling maintenance of intestinal homeostasis through tight junction protein regulation [42].

One of the greatest successes of modern medicine has been the discovery of vaccines. Vaccines have eradicated many infectious diseases. Vaccines have saved millions, possibly billions, of lives. Yet many vaccine recipients do not get adequate protection from infection. Unfortunately, our understanding of the causes of interindividual variation in immune response resulting from vaccination is woefully inadequate. While various factors, such as age at vaccination, number of doses, amount per dose, nutrition, presence of other pathogens and commensals, surely contribute to the variability in immune response, it has also been recognized that the genetic background of a vaccine recipient plays a major role in modulating AR to a vaccine. In the past decade or so, a large number of studies have been carried out to associate variations in genetic backgrounds of vaccine recipients to their ARs to the vaccine. These studies have not only resulted in many association discoveries, but have also yielded insights into mechanisms of response. We conducted this study using an injectable vaccine for typhoid that is known to have a high level of primary vaccine failure, and an oral vaccine for cholera for which hardly anything is known regarding immune response induced by the vaccine. We have found significant evidence of genes and pathways, belonging to both innate and adaptive systems, which are associated with the modulation of AR to these vaccines. Even though traditional wisdom indicates that both vaccines should act as T-cell-independent antigens, our findings reveal that the vaccines induce AR using different pathways. However, we note that these two vaccines have other differences, notably (i) their routes of administration are different and (ii) the typhoid vaccine is a single-dose vaccine, whereas the cholera vaccine is a two-dose vaccine. The latter difference may be critical in activating different pathways for mounting immune response.

It is clear from our studies that host genomics plays a significant role in modulating immune response to vaccines. Understanding these genomic correlates will help identify individuals who may require larger doses of vaccines to mount an adequate protective response, or even to formulate better vaccines by possibly using small synthetic peptides homologous to sequences of relevant genes as adjuvants.

Ethical statement. This study was approved by the Institutional Ethics Committee of the TCG-ISI Centre for Population Genomics, Kolkata; Health Ministry Screening Committee, Government of India, New Delhi and the Drug Controller General of India, New Delhi. Recruitment, vaccination and biospecimen collection were done with individual written informed consent. The study was approved by the institutional ethics committee.

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Competing interests. I have no competing interests.

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