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RESEARCH ARTICLE

Genetic determinants of immune-response to a polysaccharide vaccine for typhoid

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Abstract Differences in immunological response among vaccine recipients are determined both by their genetic differences and environmental factors. Knowledge of genetic determinants of immunological response to a vaccine can be used to design a vaccine that circumvents immunogenetic restrictions. The currently available vaccine for typhoid is a pure polysaccharide vaccine, immune response to which is T-cell independent. Little is known about whether genetic variation among vaccinees associates with variation in their antibody response to a polysaccharide vaccine. We conducted a study on 1,000 individuals resident in an area at high-risk for typhoid; vaccinated them with the typhoid vaccine, measured their antibody response to the vaccine, assayed >2,000 curated SNPs chosen from 283 genes that are known to participate in immune-response; and analyzed these data using a strategy to (a) minimize the statistical problems associated with testing of multiple hypotheses, and (b) internally cross-validate inferences, using a half-sample design, with little loss of statistical power. The first stage analysis, using the first half-sample, identified 54 SNPs in 43 genes to be significantly associated with immune response. In the second-stage, these inferences were cross-validated using the second half-sample. First-stage results of only 8 SNPs (out of 54) in 7 genes (out of 43) were cross-validated. We tested additional SNPs in these 7 genes, and found 8 more SNPs to be significantly associated. Haplotypes constructed with these SNPs in these 7 genes also showed significant association. These 7 genes are *DEFB1*, *TLR1*, *IL1RL1*, *CTLA4*, *MAPK8*, *CD86* and *IL17D*. The overall picture that has emerged from this study is that (a) immune response to polysaccharide antigens is qualitatively different from that to protein antigens, and (b) polymorphisms in genes involved in polysaccharide recognition, signal transduction, inhibition of T-cell proliferation, pro-inflammatory signaling and eventual production of antimicrobial peptides are associated with antibody response to the polysaccharide vaccine for typhoid.

Keywords Polysaccharide antigen · Antibody · Immune system · Half-sample design · Single nucleotide polymorphism · Association · Non-cognate T-cell

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Introduction

Vaccination is one of the most effective public health tools available to prevent and control the spread of infectious diseases. However, the immune response induced by a vaccine often varies between individuals, implying that a

vaccine may not afford equal protection to all vaccinees. The extent of variation in immune response has been extensively studied for some vaccines. Primary failure, as assessed by post-vaccination antibody levels, occurs in a significant proportion of vaccinees; for example, 2–10% for measles vaccine (Poland 1998; Poland et al. 1999), 5–20% for hepatitis B vaccine (Milich and Leroux-Roels 2003; Zuckerman 1996), and 23–40% for typhoid vaccine (Gupta et al. 2008; Sur et al. 2009).

Immunological mechanisms that are responsible for protection against pathogens are often unknown. Measuring clinical or microbiological protection conferred by a vaccine is best done by an artificial or a natural challenge, which is rarely possible for ethical or practical reasons. Vaccine responses are, therefore, commonly monitored by measuring humoral antibody responses and sometimes cell-mediated immune responses. Differences in antibody response (AR) among vaccinees are partly due to environmental factors, such as presence of maternal antibodies, nutrition—in particular vitamin A deficiency—or other infections, and partly due to genetic differences among the vaccinees (Kimman et al. 2007; Poland and Jacobson 1998; van Lovern et al. 2001). Twin studies have shown high and statistically significant heritability for AR to measles (89%), mumps (39%), rubella (46%), hepatitis B (61–77%), oral polio (60%), tetanus (44–64%), diphtheria (49%), *Haemophilus influenzae* type B [Hib] (51%), and other vaccines (Kimman et al. 2007). Past linkage and association studies conducted to study genomics of vaccine-induced AR have revealed many interesting results. Polymorphic variants in HLA genes have been found to be associated or linked with AR to hepatitis B vaccine (Kruskall et al. 1992), influenza vaccine (Gelder et al. 2002; Poland et al. 2008a), measles vaccine (Poland 1998; Poland et al. 2008b), and BCG tuberculosis vaccine (van Eden et al. 1983), among others. However, non-MHC or non-HLA genes—IL1B, IL4R, IL6, IL10 and TNF—have also been found to be independently associated with response to hepatitis B vaccine (Wang et al. 2004). Interestingly, although measles, mumps and rubella vaccines are all attenuated live vaccines that are administered simultaneously, the responses induced by these vaccines are influenced differently by host genetics (Kimman et al. 2007).

Vaccine immunogenicity is determined in part by the chemical and physical nature of microbial antigens and adjuvants, and also by the genetic make-up of vaccine recipients (Pulendran et al. 2001). The rationale and value of so-called "immunogenetic profiling" has been reviewed (Jin and Wang 2003); knowledge of key immunogenetic associations can be used to design a vaccine that circumvents immunogenetic restrictions. Most available vaccines comprise microbial or added protein components. Insight into the impact of genetic variation in vaccinees on AR has also been obtained in some detail (Kimman et al. 2007) for these vaccines.

Another class of vaccines is polysaccharide (PS) vaccines. PS vaccines are a unique type of inactivated subunit vaccine composed of long chains of sugar molecules that make up the surface capsule of certain bacteria, such as *Salmonella enterica* serotype typhi (*Salmonella typhi*). Currently, pure PS vaccines that are available include pneumococcal, meningococcal and typhoid vaccines. The essential feature of immune response to a pure PS vaccine is that it is typically T-cell independent, which means that a PS vaccine stimulates B-cells and non-cognate T-cells without the assistance of cognate T cells (Vos et al. 2000; Snapper 2006). Little is known about the role of genetic variation in vaccinees on antibody response to PS vaccines.

Worldwide, typhoid fever affects 17 million people annually, causing 600,000 deaths. It is caused by *S. typhi* that is an obligate parasite and has no natural reservoir outside of human. It is a gram-negative enteric bacillus and multi-organ parasite that inhabits the lymphatic tissues of small intestine, liver, spleen and bloodstream of infected individuals. Over 100 strains of this parasite—that differ in levels of virulence—have been isolated. It enters uninfected individuals via the fecal-oral route from infected individuals. Therefore, individuals living under unhygienic conditions—particularly in areas with open sewage, public latrines and unclean drinking water—are most vulnerable to typhoid infection and therefore in the greatest need of vaccination. *Salmonella typhimurium*, that causes murine typhoid, causes enteritis in humans characterized by self-limited fever and diarrhea and, in some cases, dysentery; symptoms that are rarely observed with *S. typhi* infection. *S. typhi* has an outer capsule, Vi polysaccharide, that is absent in *S. typhimurium.* Vi is a polymer of α-1,4-galacturonic acid with an *N*-acetyl at position C-2 and variable *O*-acetylation at C-3 (Szu et al. 1991). The expression of this molecule correlates with virulence, resistance to phagocytosis and resistance to complement-mediated killing (Looney and Steigbigel 1986). Therefore, the Vi PS has been used as a vaccine to protect individuals from *S. typhi* infection. The objective of this study was to quantify the extent of variation in antibody response to a widely used Vi PS vaccine for typhoid and to discover associations of polymorphisms in candidate genes of vaccinees with antibody response. To our knowledge, this is the first large-scale study on genomics of immune response of a polysaccharide vaccine.

Materials and methods

Institutional ethical approvals were taken from all collaborating institutions before initiation of this study. Written

informed consent was taken from each recruited participant. Approval of the Drug Controller General of India was taken before administration of the vaccine to the study participants. Dispatch of aliquots of serum samples collected in this study to the collaborating laboratories in the USA was approved by the Health Ministry Screening Committee, Government of India.

Study participants

Individuals ($n = 1{,}000$), unrelated at least to the first-cousin level, aged 12 years or older, inhabiting a socio-economically depressed locality of Kolkata (formerly, Calcutta), India, were recruited into this study. The residents belonged to two maritally isolated, religious groups, Muslim and Hindu. The Muslims of this locality are mostly religious converts, during the last 100 years, to Islam from Hinduism. The individuals recruited into this study are demographically (age-group, gender and religion) representative of the entire locality, since care was taken to recruit into age × gender × religion subgroups by a probability proportional to size sampling scheme, with population sizes of subgroups having been determined by us through an initial complete-enumeration demographic survey of the locality (comprising ~9,950 individuals). The following exclusionary criteria were used, based on self-report, to recruit an individual into this study: that (a) she/he had not had fever lasting for more than three consecutive days in the past 1 year, and (b) she was not pregnant or lactating.

Vaccination and collection of blood samples

Each study participant was injected, intramuscularly, with a 0.5 ml single-dose injectable vaccine, containing 25 μg of the cell surface Vi polysaccharide extracted from *S. typhi* Ty2 strain with the excipients sodium chloride, sodium dihydrogen phosphate dihydrate, disodium phosphate dihydrate, phenol and water for injection. This vaccine is marketed in India as Typherix® (manufactured by GlaxoSmithKline, Inc.), and is widely used in Asia, Africa and South America, and approved for use elsewhere, including the European Union and the USA.

From each study participant, a blood sample was collected immediately prior to vaccination. Blood samples were also collected 3- and 28-days post-vaccination. Serum and DNA were isolated from these samples, using standard protocols and QiaGen columns.

Antibody response assay

A bead assay was performed using the Bio-Plex (Bio-Rad, Hercules, CA) platform. The Vi PS antigen (supplied by Fina Biosolutions, Rockville, MD, USA) was conjugated to Bio Rad beads [COOH (028)] at a concentration 8.33×10^6 beads/ml in a total volume of 1.50 ml. About 50 μl of human serum was 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 1:3,200; Day-28 serum diluted 1:200, 1:1,600 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 (1:5,000 in human serum diluent) by incubation for 30 min. Following the final wash steps, the beads were analyzed 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 arbirtrary value of 200 ELISA units (EU) per ml. The responses in the individual subjects were compared to the standard curve to calculate anti-Vi IgG EU/ml in the Day-0 and Day-28 serum.

Candidate genes and SNPs

We selected 283 autosomal genes from immunological pathways as candidates for this association study. SNPs from these genes, including about 2 and 1 kb upstream and downstream regions respectively, were chosen from the HapMap database (http://www.hapmap.org). The choice of SNPs was done using a statistical protocol that took into account differences in LD patterns and allele frequencies in the four HapMap populations. The statistical protocol provided a ranking of the "potential informativeness" of SNPs within each gene cataloged in the HapMap database, from which a subset of maximally informative SNPs was chosen. Further details are provided in Supplementary Text S1 and the list of genes and SNPs are provided in Table 1 and Table S1. Genotyping was done using Sequenom (55.7% of assayed SNPs) and Illumina (remaining 44.3% of SNPs) platforms.

Statistical analysis

Antibody response (AR) to vaccination was measured as the difference between 28-day post-vaccination and pre-vaccination antibody levels (EU/ml). Values of AR were $\log_{10}$-transformed (*log*AR) to induce normality to the frequency distribution (Fig. 1) that is essential for using standard parametric statistical methods for hypothesis testing. Curation of genotype data included removal of (a) loci with <90% call rate (8.9% of loci), (b) non-polymorphic loci with MAF <0.05 (24.7% of loci with call rate >90%), (c) significantly deviant from HWE (4.2% of polymorphic loci), (d) individuals with <75% of loci with valid genotype calls, (e) individuals on whom antibody

Table 1 Numbers of genes in various functional classes included in this study

GO category[a]	Molecular function	No. of genes
GO:0004871	Signal transducer activity	86
GO:0005102	Receptor binding	44
GO:0001664	G-protein-coupled receptor binding	41
GO:0003676	Nucleic acid binding	21
GO:0000166	Nucleotide binding	19
GO:0001584	Rhodopsin-like receptor activity	16
GO:0005488	Binding	12
GO:0003824	Catalytic activity	7
GO:0004857	Enzyme inhibitor activity	6
GO:0003823	Antigen binding	3
GO:0001871	Pattern binding	3
GO:0001530	Lipopolysaccharide binding	2
GO:0008009	Chemokine activity	1
GO:0001948	Glycoprotein binding	1
GO:0015457	Auxiliary transport protein activity	1
GO:0032393	MHC class I receptor activity	1
GO:0003774	Motor activity	1
GO:0032395	MHC class II receptor activity	1
GO:0001532	Interleukin-21 receptor activity	1
GO:0005125	Cytokine activity	1
GO:0000287	Magnesium ion binding	1
GO:0005048	Signal sequence binding	1
GO:0005515	Protein binding	1
GO:0001540	Beta-amyloid binding	1
GO:0005215	Transporter activity	1
GO:0003712	Transcription cofactor activity	1
Miscellaneous (GO category unavailable; information obtained from UNIPROT[b] database)	Mediation of inflammation and angiogenesis	1
	Peptide binding	1
	Antimicrobial activity	3
	T-cell activation	1
	Defense response with MHC	1
	Regulation of inflammatory responses	1
	Putative immune function (Gene: *HCG9*; lies within MHC class I region)	1
Total		283

[a] GO (Gene Ontology) category was obtained from gene2go file of NCBI Entrez Gene database (ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/)

[b] (http://www.uniprot.org/)

response could not be properly assayed (8 of 1,000 individuals), and (f) individuals showing cryptic relationships (>80% of loci with identical genotypes; 6 pairs of individuals satisfied this criterion, one individual from each pair was randomly chosen for final analysis). After data-curation, the final data set comprised genotypes of 984 individuals at 2,040 SNPs in 283 genes. Table 1 provides the numbers of genes in different classes of molecular function; the complete list of genes, SNPs selected from these genes and other relevant information are provided in Supplementary Table S1. Analysis of Variance (ANOVA) was performed to test equality of mean values of *log*AR among subgroups of vaccinees.

In conducting detailed statistical analyses of these data, we deviated from "standard practice" (Wellcome Trust Case Control Consortium 2007; Wellcome Trust Case Control Consortium et al. 2007; Burgner et al. 2009; Samani et al. 2007; Baranska et al. 2008; Hennig et al. 2008) and adopted a novel strategy. First, we did not classify the vaccinees as "responders" and "non-responders", because this would involve defining an arbitrary threshold on values of antibody response to enable classification. Instead, we

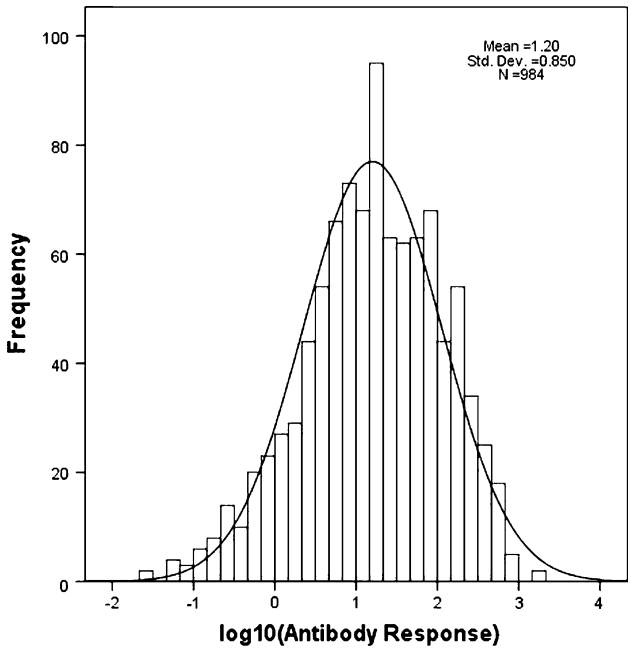

Fig. 1 Frequency distribution of $\log_{10}$-transformed values of antibody response among vaccinees

sorted the vaccinees by ascending order of their antibody response and grouped the vaccinees into 5 pentile (20 percentile) groups. Second, we posited that if indeed an allele at a locus was positively (or, negatively) associated with antibody response, then its frequency among vaccinees should monotonically increase (or, decrease) from those belonging to the lowest pentile group of antibody response to those belonging to the highest pentile group. In other words, the correlation coefficient between the minor allele frequency (MAF) at the locus and mean AR of vaccinees belonging to the pentile groups should be statistically significant. Third, in genomic studies involving a large number of associated loci, correction for multiple testing—especially if the loci are in significant linkage disequilibrium (LD); that is, non-independent—remains a major statistical problem (Conneely and Boehnke 2007; Han et al. 2009). To circumvent this, for single-locus association analysis, we reduced the number of tests by selecting from each gene only the highly informative and minimally associated SNPs, initially selecting the SNP located at the most 5′ end of the genomic region covered for the gene. This strategy resulted in the choice of 469 SNPs from the genes under consideration. (The data on the remaining loci were unused in the single-locus association analysis, but were used for haplotype association analysis, as explained later.) Fourth, cross-validation of findings of an association study is often a problem. In addition, replication of a study similar to the present one is particularly difficult because it entails the ethical choice of a population in which individuals will benefit from the vaccination to be provided. If the results are cross-validated in a different population, then in the event of discordance of results with the initial study it is difficult to ascertain whether population differences were the cause of the observed discordance. To avoid these vagaries, we generated an internal cross-validation sample, by randomly splitting the 984 vaccinees into two half-samples, of $\sim$500 vaccinees in each half-sample (with $\sim$100 vaccinees in each of the 5 pentile groups based on antibody response within each half sample). While this strategy in which $\sim$500, instead of $\sim$1,000, vaccinees are considered in the initial association analysis results in some loss of statistical power, our simulation studies showed that the loss of power is only 3–14% depending on the value of the correlation coefficient between MAF and mean AR (results not shown). The reason why the loss of statistical power is small is because a sample of size $\sim$500 individuals (included in each half-sample) is large. The small loss of statistical power is more than offset by the gain in ability to cross-validate results using the same population sample. The strategy is presented as a flow-chart in Fig. 2.

Test of significance of the correlation coefficient (r) between MAF and mean antibody response was carried out by a permutation test. Genotypes of individual vaccinees at a locus were randomly permuted and the correlation coefficient was computed in this permuted sample. This procedure was repeated 1,000 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 FDR procedure (Benjamini and Hochberg 1995) was used to identify statistically significant values of r at the 5% level of significance.

We have also revalidated our inferences using the 'standard' procedure for association analysis, in which the null hypothesis tested is that the mean *log*AR values among the genotype classes are equal. This null hypothesis is tested using ANOVA. All significant allelic associations with *log*AR detected using the procedures described above were also validated using genotype data using PLINK (Purcell et al. 2007), Version 1.06 (http://www.pngu.mgh.harvard.edu/~purcell/plink/).

To summarize our analytical strategy, we first tested—in a random sample of 50% of our data (half-sample 1)—whether the minor allele frequency at a locus increases (or decreases) with increasing antibody response among groups of vaccinees using correlation analysis, and data-permutation and false discovery rate procedures for assessing significance of the estimated correlation coefficient. For those SNPs that showed significant correlation, we cross-validated the results in the remaining 50% of our data (half-sample 2), using the same methodology

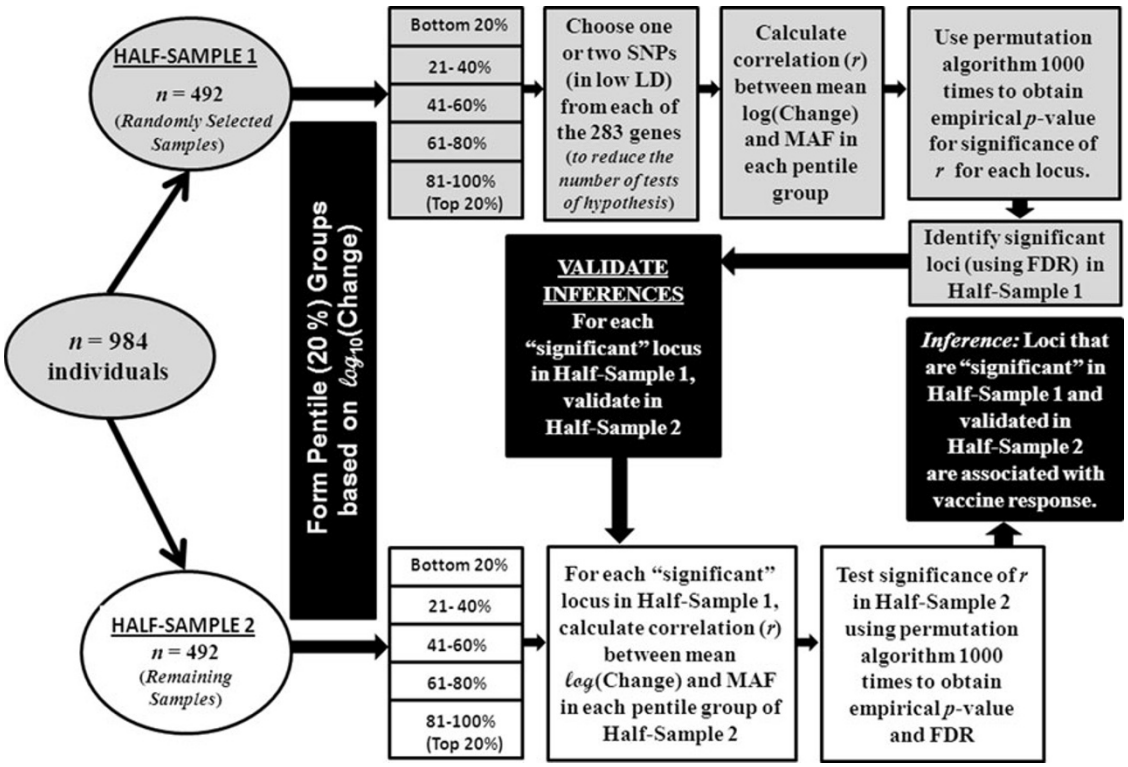

Fig. 2 Flow-chart describing the strategy used in the conduct of statistical data analyses

(correlation, data-permutation and FDR analyses). Finally, those SNPs for which inferences could be cross-validated, we revalidated the inferences using a 'standard' approach of testing equality of mean antibody-response among genotypes by ANOVA.

Haplotypes were inferred using PHASE (Stephens et al. 2001; http://www.stat.washington.edu/stephens/phase.html). Haplotype association analysis was performed using the permutation algorithm described above for single SNP markers.

Results

Demographic characteristics of vaccinees

Among the vaccinees ($n = 984$), 62.7% were Muslim and 37.3% were Hindu; 46.4% were male and 53.6% were female. The mean age of the vaccinees was 33.7 ± 0.4 years. The male vaccinees (mean age = 34.7 years) were, on the average, 2 years older than the female vaccinees (mean age = 32.7 years); this difference was statistically significant ($P = 0.015$). However, the mean ages of the vaccinees did not differ significantly by religion (mean age of Muslim and Hindu vaccinees were, respectively, 33.6 and 33.8 years; $P = 0.76$).

Pre- and post-vaccination antibody levels and antibody response

Mean antibody levels (in EU/ml) prior to vaccination were low, but were high on the 28th day post-vaccination (Table 2). The antibody levels pre- or post-vaccination were not significantly influenced by gender or religion.

Enormous variation in both pre- and 28-day post-vaccination antibody levels was observed. Table 3 provides the descriptive statistics for the three variables (Days-0 and

Table 2 Pre- and post-vaccination antibody levels (EU/ml) among vaccinees and their antibody response, classified by gender and religion

	Pre-vaccination	Post-vaccination	Antibody response
Gender			
Male	1.77 ± 0.67	61.34 ± 5.10	59.57 ± 5.00
Female	1.38 ± 0.27	77.45 ± 7.03	76.06 ± 6.98
P-value*	0.185	0.088	0.100
Religion			
Muslim	1.78 ± 0.53	77.48 ± 6.29	75.70 ± 6.23
Hindu	1.21 ± 0.23	57.34 ± 5.51	56.14 ± 5.42
P-value*	0.582	0.245	0.302

* *P*-values were calculated after $\log_{10}$-transformation of the data to induce normality of the frequency distributions

Table 3 Ranges and descriptive statistics of pre- and post-vaccination levels and antibody response (AR) among vaccinees belonging to the five pentile groups based on pre-vaccination levels

Day-0 pentile group	Variable	Mean (EU/ml)	SD (EU/ml)	Minimum (EU/ml)	Maximum (EU/ml)
1 ($n = 196$)	Day-0	0.026	0.014	0.000	0.050
	Day-28	7.218	10.065	0.045	68.173
	AR	7.193	10.064	0.031	68.136
2 ($n = 197$)	Day-0	0.081	0.020	0.050	0.120
	Day-28	21.637	56.840	0.108	691.270
	AR	21.556	56.839	0.056	691.211
3 ($n = 197$)	Day-0	0.210	0.060	0.121	0.328
	Day-28	40.696	66.614	0.201	514.707
	AR	40.486	66.604	0.067	514.521
4 ($n = 197$)	Day-0	0.607	0.208	0.329	1.079
	Day-28	74.254	96.472	0.698	717.317
	AR	73.647	96.440	0.294	716.404
5 ($n = 197$)	Day-0	6.891	23.264	1.084	293.561
	Day-28	205.719	234.984	2.769	1,777.819
	AR	198.827	234.098	0.341	1,775.567

28 antibody levels, and antibody response (AR) = Difference between Day-28 and Day-0 antibody levels) among vaccinees belonging to the five pentile groups based on Day-0 antibody levels. Irrespective of the pre-vaccination antibody level, vaccinees showed considerable variation in post-vaccination levels or antibody response (see also Supplementary Figure S1). The correlation between pre- and post-vaccination antibody levels was low (0.188), but significant ($P < 0.0005$). Among the vaccinees, the observed differences in the *log*AR (Fig. 1) could not be explained by variation in age ($P = 0.915$), or by gender ($P = 0.100$) or religious group ($P = 0.302$) differences (Table 2) of the vaccinees. Therefore, we did not stratify the vaccinees by age, gender or religious group for further statistical analyses.

Association analysis

After data-curation, data were available on 984 individuals pertaining to 2,040 SNPs in 283 genes. Of these 2,040 SNPs, the difference in the MAFs between Muslim and Hindu vaccinees was not statistically significant at the 5% level for any locus, after correcting for multiple-testing (Supplementary Figure S2). Thus, even though the vaccinees belonged to two maritally isolated religious groups, there was no genetic stratification or sub-structuring among the vaccinees. The vaccinees were randomly grouped into two half-samples; the demographic characteristics of the vaccinees in these two half-samples were similar and statistically non-significant (Supplementary Table S2 and Supplementary Figure S3). Vaccinees belonging to each half-sample were grouped into 5 pentile groups based on their AR values; that is, groups defined on the basis of AR values sorted in ascending order, with each group comprising approximately 20% of the total sample. The mean values of AR among vaccinees belonging to the pentile groups within half-samples are presented in Table 4. Between the two half-samples, while the mean values of AR were not statistically significant for the first three pentile groups, there were significant differences for the fourth and fifth pentile groups. These differences arose from a few individuals who showed high AR in either of the two half-samples (Supplementary Figure S3), as is evident from the large standard deviations of AR for these two pentile groups. While the increase in mean AR from the first to the third pentile groups is gradual, there is a sharp increase from third to the fourth pentile group and also from the fourth to the fifth pentile group. The mean values of *log*AR and the MAF for each locus were computed for vaccinees belonging to each pentile group within

Table 4 Mean ± SD antibody response (EU/ml) among individuals belonging to various pentile classes

Half-sample	Pentile class (n in each half-sample)				
	1 ($n = 98$)	2 ($n = 98$)	3 ($n = 98$)	4 ($n = 98$)	5 ($n = 100$)
1	1.28 ± 1.00	6.50 ± 1.97	17.11 ± 4.46	63.80 ± 26.66	308.44 ± 252.31
2	1.39 ± 0.97	6.49 ± 2.24	18.08 ± 4.92	47.95 ± 12.52	205.31 ± 160.59
P-value	0.450	0.983	0.150	<0.0009	0.001

Table 5 List of 54 SNPs in 43 genes, along with the corresponding correlation coefficient between minor allele frequency and antibody response, that showed significant association with antibody response in half-sample 1

Chromosome no.	Gene	SNP rs #	Corr. coeff. (r)	Chromosome no.	Gene	SNP rs #	Corr. coeff. (r)
1	*TNFRSF8*	12736809	−0.94	4	*IRF2*	12512614	−0.96
1	*TNFRSF1B*	505844	−0.97	6	*BAT2*	2736172	−0.91
1	*IL12RB2*	7555183	−0.99	6	*HLA-DOB*	2859579	−0.99
1	*TGFBR3*	1017956	−0.94	6	*IL22RA2*	13197049	0.92
1	*XCL1*	6700487	0.91	6	*CCR6*	1571878	−0.96
1	*PTGS2*	2745557	0.93	8	*DEFB1*	2978873	0.98
1	*IRF6*	2073486	−0.93	8	*DEFB1*	2977772	0.91
1	*NLRP3*	12565738	−0.92	8	*IKBKB*	3747811	−0.95
1	*NLRP3*	10754558	0.98	9	*PAX5*	7031673	−0.91
2	*IL1R2*	13022757	−0.93	10	*IL2RA*	10905668	0.98
2	*IL1R2*	4850993	−0.95	10	*ITGB1*	10827164	−0.95
2	*IL1R2*	3218848	0.98	10	*CXCL12*	10900030	0.96
2	*IL1R2*	2110562	−0.92	10	*MAPK8*	12358297	−0.95
2	*IL1RL1*	10208293	0.95	10	*CHUK*	2230804	−0.91
2	*IL18R1*	1362348	0.92	11	*CD59*	3181274	−0.94
2	*CTLA4*	231779	−0.99	11	*CD44*	112762	−0.97
3	*IL5RA*	2290610	−0.98	11	*IL10RA*	4252306	0.93
3	*CMTM8*	4132830	−0.96	11	*TIRAP*	8177352	0.98
3	*CMTM7*	17029530	−0.97	13	*IL17D*	1888001	0.94
3	*CD80*	491407	−1.00	13	*TNFRSF19*	7338328	−0.98
3	*CD80*	2228017	−0.97	16	*IL4R*	4787423	0.96
3	*CD80*	2222631	−0.93	17	*CSF3*	2227321	−0.95
3	*CD80*	16829988	−0.99	17	*MAP2K6*	12946388	−0.95
3	*CD86*	2681411	0.97	21	*ITGB2*	235326	0.99
3	*CD86*	17203439	0.95	22	*MAPK1*	743409	−0.93
4	*TLR1*	5743572	0.93	22	*MAPK1*	2283792	−0.98
4	*CXCL13*	171388	−0.94	22	*MAPK1*	3788332	−0.94

each half-sample. The differences in *log*AR between consecutive pentile groups were statistically significant, within each half-sample and for the total sample. The differences in MAFs between half-samples for the 5 pentile groups are presented in Supplementary Figure S4; none of the differences, corrected for multiple-testing, was statistically significant at the 5% level. To partially circumvent the statistical problems associated with multiple-testing, a subset of 469 (out of 2,040) 'highly informative and minimally associated' SNPs was chosen, with at least one SNP in each of the 283 genes (see Materials and Methods section for further details), for an initial association analysis. The correlation coefficient (r) between MAF and mean *log*AR of vaccinees belonging to the pentile groups of half-sample 1 was computed for each of the 469 loci selected for the association analysis. Of these 469 SNPs, only 54 (11.5%) SNPs in 43 genes showed significant correlation (P-values were estimated by data-permutation), at the 5% level (using multiple-testing correction by FDR method), between MAF and mean *log*AR of vaccinees belonging to the pentile groups (Table 5).

Cross-validation analysis

The inference that 54 SNPs were significantly associated with AR was cross-validated using the data on vaccinees belonging to half-sample 2—in which, as expected, the demographic characteristics were similar to those of half-sample 1(Supplementary Table S2 and Supplementary Figure S3). The same procedure of assessing the significance of relationship between *log*AR and MAF was used for each of the 54 SNPs considered for cross-validation. Of these 54 SNPs, significant association was found only for 8 SNPs—all intronic—in 7 genes (Table 6). Figure 3 shows that there is a near monotonic increase (for 6 SNPs) or decrease (for 2 SNPs) of MAF with increasing AR.

Significance of the other assayed SNPs in genes showing cross-validated association

To avoid vagaries of multiple-testing, for the association analysis we selected a subset of assayed SNPs that were not in strong LD, and identified 8 associated SNPs in 7 genes (Table 6). We next sought to examine whether any of the remaining assayed SNPs in these 7 genes also showed significant association. In the cross-validated gene, *CTLA4*, only one SNP was selected for genotype assay, since the size of the gene is small. In the remaining 6 genes (Table 6), 100 SNPs were assayed (Table S1), of which 7 were found to be significantly associated with AR and were cross-validated. Among the additional 93 assayed SNPs in these 6 genes, 8 showed significant association (Table 6). These 8 SNPs were also intronic. Quantitative trait (antibody response) association analysis with genotypes was performed for these 16 SNPs; that is, we tested the null hypothesis that the mean values of *log*AR among vaccinees belonging to the three genotypes in the complete data set (half samples 1 and 2 pooled) were equal. This was carried out by ANOVA using PLINK (http://www.pngu.mgh.harvard.edu/~purcell/plink/); all differences were found to be significant (Table 6) at the 5% level, implying that mean antibody responses were significantly different among genotypes.

Haplotype analysis

With the exception of *CTLA4*, for each of the remaining 6 genes (*IL1RL1*, *CD86*, *TLR1*, *DEFB1*, *MAPK8* and *IL17D*) multiple SNPs were assayed, but were not included in the initial analyses to reduce the number of tests. In these 6 genes, we carried out association analyses with all the assayed SNPs, and found some of these additional SNPs to be significantly associated with AR. The additional SNPs that were significantly associated belonged to 3 genes—*IL1RL1*, *TLR1* and *DEFB1* (Table 6). For these 3 genes in which multiple SNPs showed significant association with antibody response, we estimated the frequency of the haplotype comprising minor alleles at the SNP loci that were found to be significantly associated (Table 6). For each of these genes, the signs of the correlation coefficient between MAF and mean *log*AR of pentile groups for all SNPs that were significantly associated were identical (data not shown). We then estimated the strength of association between the frequencies of these haplotypes and mean *log*AR of vaccinees belonging to the pentile groups using the total sample ($n = 984$). Essentially, the nature of and strength (significant at 5% level) of relationship remained unaltered (Fig. 4) from that found in the cross-validation analysis (Fig. 3). Thus, haplotypes based on SNPs in these genes also showed significant association with AR.

Discussion

Salmonella typhi, that causes typhoid fever, is encapsulated by a polysaccharide (PS), Vi PS. The expression of Vi PS correlates with virulence and resistance to nonoxidative killing and phagocytosis (Looney and Steigbigel 1986). Infection with encapsulated bacteria can be prevented by vaccination with capsular polysaccharides. A Vi PS vaccine for typhoid is marketed and used in many countries, including India. Since the transmission of the typhoid pathogen is through the oral-fecal route, typically the areas of endemicity of typhoid are also areas where there is low level of personal and public hygiene. Although it is in such areas where a vaccination program against typhoid is likely to have a major public-health impact, residents of such areas are often exposed to *S. typhi* and other co-infections prior to vaccination. Indeed, several studies have reported that 19–58% percent of subjects that live in endemic areas involved in Vi polysaccharide vaccine studies develop protective levels (1 μg/ml) of anti-Vi antibodies without vaccination (Klugman et al. 1996; Keddy et al. 1999; Panchanathan et al. 2001). These observations suggest that natural, environmental exposure to *S. typhi* has the potential to induce protective levels of serum, anti-Vi antibodies. Post-vaccination immune response is likely to be affected by prior exposure. Participants in this study were chosen from a high-risk area for typhoid infection. Using appropriate criteria, we excluded individuals who may have been infected with *S. typhi* at least a year prior to their recruitment in this study, thereby minimizing, to the extent feasible, the confounding effect of immune response due to prior exposure with that due to vaccination. Our data have revealed that post-vaccination antibody levels do not strongly correlate with pre-vaccination levels. This indicates that the vaccine can induce a strong antibody response even if a vaccinee has a high pre-vaccination level of antibody and the vice versa.

Immune response to polysaccharide antigens is qualitatively different from that to protein antigens. Typically, it is T-cell independent. T-cell independent antigens are not consistently immunogenic in children <2 years of age (Chelvarajan et al. 2005). The Vi polysaccharide vaccine represents a T-independent antigen that does not generate antigen-specific $CD4^+$ T cell help. Without $CD4^+$ T cell help, long-term memory B cells are not generated. This explains the need to revaccinate with the Vi polysaccharide vaccine every 2–3 years (Whitaker et al. 2009). However, T-cell-independent type 2 (TI-2) antigens, such as Vi PS, can also stimulate the mature B cells to induce antibody production through non-cognate T-cells (Obukhanych and Nussenzweig 2006) and antibody response to Vi PS vaccine has been shown to be correlated with the number of $CD4^+$ T lymphocytes (Kroon et al. 1999).

Table 6 Details of 8 SNPs in 7 Genes that showed significant cross-validated association with antibody response, and of other assayed SNPs in those genes that showed significant association in both half-samples

Gene	Chromosome no.	Total no. of SNPs assayed[a]	No. of "significant" SNPs	Details of "significant" SNPs			*r* In half-sample 1 ($n = 492$)[d]	*r* In half-sample 2 ($n = 492$)[d]	*P*-value using PLINK in the total sample[e] ($n = 984$)
				rs #	nt Position	SNP location within gene (mRNA transcript no. in NCBI database)			
IL1RL1	2	13	3	4142132[b]	102303914	Intron 1 (NM_016232.4)	0.96	0.94	0.011
				1997466[b]	102317899	Intron 1 (NM_016232.4)	0.91	0.94	0.016
				10208293[c]	102332742	Intron 10 (NM_016232.4)	0.95	0.91	0.002
CTLA4	2	1	1	**231779** [c]	204442732	Intron 1 (NM_005214.3)	−0.99	−0.93	0.028
CD86	3	17	1	**17203439**[c]	123278196	Intron 1 (NM_175862.3)	0.95	0.97	0.016
TLR1	4	24	2	55815313[b]	38481645	Intron 2 (NM_003263.3)	0.95	0.94	0.022
				5743572[c]	38481786	Intron 2 (NM_003263.3)	0.98	0.93	0.050
DEFB1	8	33	7	**2978873**[c]	6717373	Intron 1 (NM_005218.3)	0.98	0.98	0.016
				2977780[b]	6717513	Intron 1 (NM_005218.3)	0.95	0.95	0.018
				2980928[b]	6717829	Intron 1 (NM_005218.3)	0.95	0.95	0.018
				2978872[b]	6718140	Intron 1 (NM_005218.3)	0.93	0.98	0.008
				2951854[b]	6718836	Intron 1 (NM_005218.3)	0.92	0.94	0.028
				2980923[b]	6720467	Intron 1 (NM_005218.3)	0.95	0.93	0.015
				2977772[c]	6720606	Intron 1 (NM_005218.3)	0.91	0.94	0.013
MAPK8	10	7	1	**10857564**[c]	49282578	Intron 1 (NM_002750.2)	−0.95	−0.96	0.030
IL17D	13	6	1	**1888001**[c]	20183906	Intron 2 (NM_138284.1)	0.94	0.91	0.018

[a] Including assayed SNPs in 2 kb regions upstream and downstream of the gene

[b] Association significant in both Half-samples (was not included in the initial analysis of 469 SNPs)

[c] Association found to be significant in Half-Sample 1 and cross-validated in Half-Sample 2 (i.e., included in the initial analysis of 469 SNPs)

[d] *P*-values corresponding to these correlation coefficients are all <0.005, and with FDR < 0.05

[e] Essentially each *P*-value corresponds to a test of equality of mean values of *log*AR among individuals belonging to the three genotypes at the locus under consideration

Despite their T-cell independent nature, some polysaccharide antigens have been reported to activate dendritic cells and other cell types. When dendritic cells were treated with the polysaccharide from *Ganoderma lucidum* there was enhanced cell surface expression of CD80, CD86, CD83, CD40, CD54, and HLA-DR (Kroon et al. 1999). It is also known that blood dendritic cells interact with marginal zone (MZ) B cells to initiate TI-2 immune responses as polysaccharide antigens localize preferentially to these (MZ) B cells found in spleen (Achtman et al. 2009; Klouwenberg and Bont 2008). The splenic marginal zone (MZ) has been implicated as a source of circulating IgM memory B cells (Kreutzmann et al. 2003). These findings reveal a larger role for polysaccharides in immune recognition than currently appreciated.

Inter-individual variability in immune response to peptide vaccines has been documented and a number of polymorphic loci in genes related to both innate and adaptive immune systems have been found to be associated with antibody response to vaccines (Sur et al. 2009). However, little is known about genomics of immune response for polysaccharide vaccines. To our knowledge, this is the first large-scale study—involving a large number of vaccinated individuals ($\sim$1,000) and a large number of polymorphic loci (>2,000)—on genomics of immune response to a polysaccharide vaccine to prevent *S. typhi* infection. Our strategy for data analyses was chosen to minimize the possibility of false positive discovery and, with a minimal loss of statistical power, enabled internal cross-validation of results through a half-sample analytical design.

Of the 283 genes considered as candidates for this study of immune response, we have found 16 SNPs in 7 genes to show significant allelic, genotypic and haplotypic associations with immune response to the Vi PS vaccine. These 7 genes are β-defensin 1 (*DEFB1*) with 7 associated SNPs; toll-like receptor 1 (*TLR1*) and interleukin 1 receptor-like 1 (*IL1RL1*) each with 2 associated SNPs; cytotoxic T-lymphocyte-associated antigen 4 (*CTLA4*), mitogen-activated protein kinase 8 (*MAPK8*), cluster of differentiation 86—a molecule expressed on antigen-presenting cells that provide costimulatory signals necessary for T cell activation

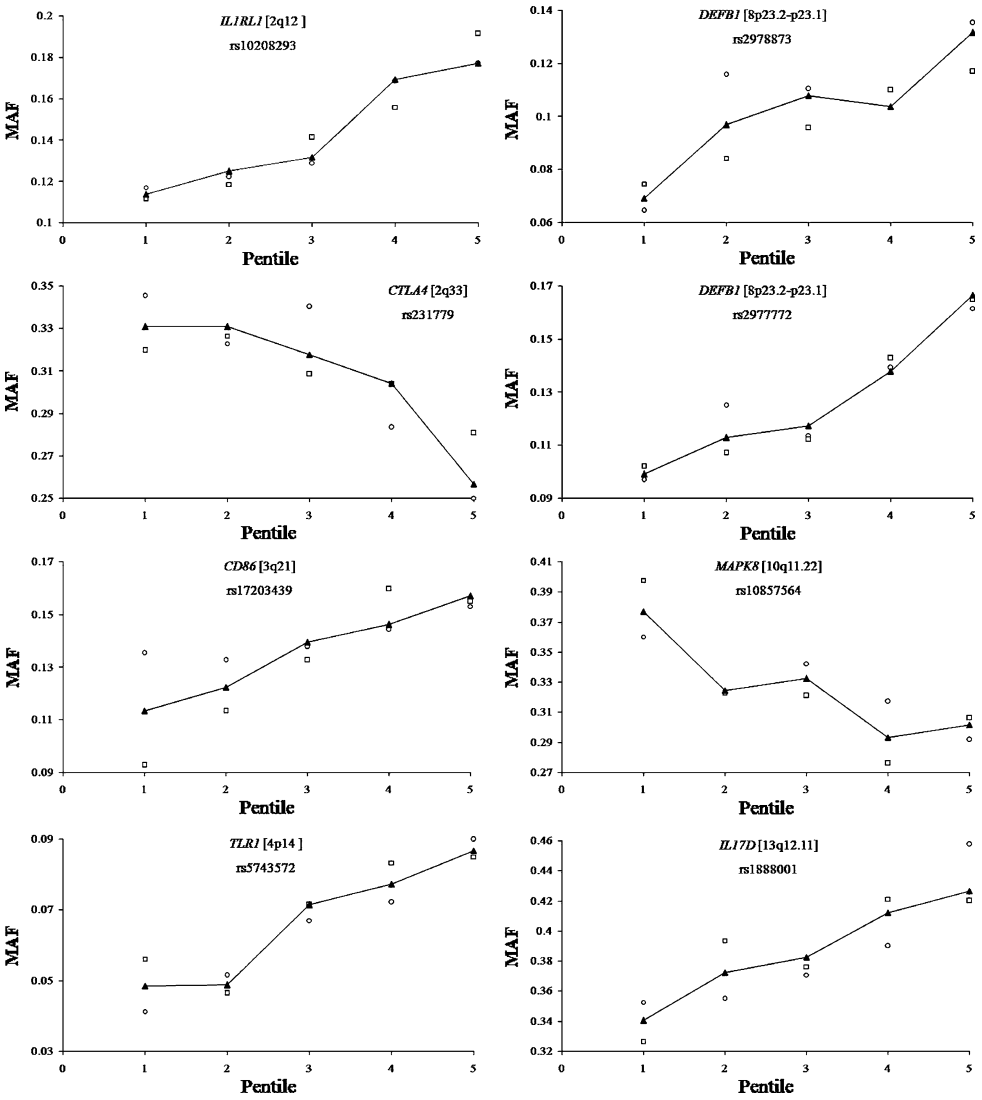

Fig. 3 Increase/decrease in minor allele frequency (MAF) from the lowest to the highest pentile group of antibody response (*open circles* pertain to half-sample 1, *open squares* to half-sample 2 and *solid triangles* to the complete data)

and survival (*CD86*) and interleukin 17D (*IL17D*) each with 1 associated SNP.

Though the role of T lymphocytes in the regulation of the antibody production to PS is obscure, anti-PS response involves non-cognate activation of TCR-nonspecific T cells by implicating a role for endogenous CD40-CD40L interactions in the human antibody response (Snapper et al. 2001). It is known that B7 family of co-stimulatory molecules, CD86 along with CD80, present on the antigen-presenting cells (APCs), interact with CD28/CTLA4 receptors on T cells to provide major non-cognate co-stimulatory signals (Khan et al. 2007). Therefore, it is not surprising that *CD86* and *CTLA4* genes showed significant association with immune response to Vi PS vaccine in the present study, indicative of a non-cognate T-cell dependence. *TLR1* showed association with the host response to the vaccine. Downregulation of TLR-mediated immune responses through diminished TLR-mediated cell signaling or expression is an important immune evasion mechanism in some bacterial pathogens (Alvarez 2005; Babu et al. 2009). Data from experiments with human colonic tissue explants indicate that the Vi antigen reduces TLR-mediated IL-8 signaling, thereby evading TLR-mediated host response triggering neutrophil infiltration in the intestinal mucosa (Wilson et al. 2008). However, it is not clear if Vi polysaccharide is recognized by TLR1 or if the *TLR1* gene influences host responses to co-infections that then indirectly influences host response to vaccination with Vi PS. All toll-like receptors have a toll-interleukin 1 domain that is responsible for signal transduction; *IL1RL1*—a gene that we have found to be significantly associated with AR induced by the PS vaccine—participates in this signaling.

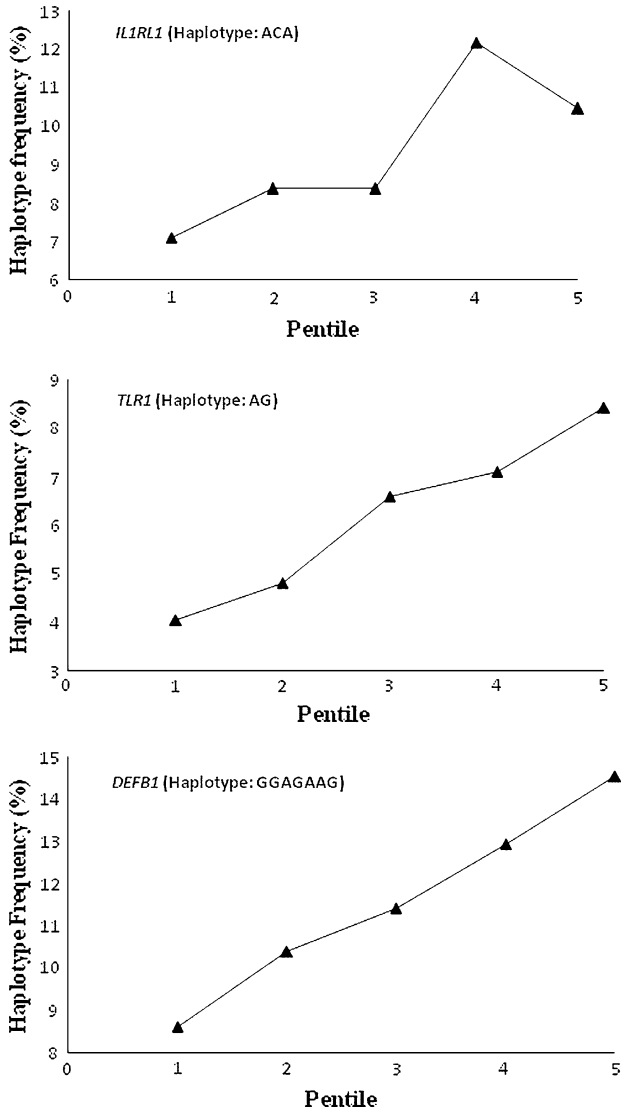

Fig. 4 Increase/decrease in haplotype frequency from the lowest to the highest pentile group of antibody response in the total sample

MAP kinases are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development; therefore, the association of a SNP in *MAPK8* with antibody response is not unexpected. There are reports demonstrating that the MAP kinase pathway may be one of the targets that is modulated by Vi to dampen early inflammatory responses in Vi polysaccharide treated intestinal epithelial cells (Caco-2; Sharma and Qadri 2004). This may explain why *MAPK8* was found to be associated with Vi polysaccharide antibody response. The involvement of a MAP kinase and *IL17D*, that stimulates the production of other cytokines, can result in pro-inflammatory signals that can induce upregulation of *DEFB1* (O'Neil et al. 1999). We have found multiple SNPs in *DEFB1* to be significantly associated with Vi polysaccharide vaccine response. Thus, the overall picture that emerges from our findings is that polymorphisms in genes involved in polysaccharide recognition, signal transduction, inhibition of T-cell proliferation, pro-inflammatory signaling and eventual production of antimicrobial peptides are associated with antibody response to the Vi polysaccharide vaccine for typhoid. However, just as co-infection with parasites (Harris et al. 2009) or other infectious agents (Sugata et al. 2008; Lin et al. 2006) can influence immunological response to a pathogen, it is possible, that antibody response to vaccination with Vi polysaccharide may be affected by co-infections or prior exposure. While it is difficult to rule out the involvement of other genes in modulation of immune response to the Vi polysaccharide vaccine, the 7 genes that we have found to be associated with host antibody response to the polysaccharide vaccine provide a foundation for elucidating the functional mechanism of the products of these genes in host response to the polysaccharide vaccine. However, we emphasize that the evaluation of genes that influence host antibody response to the Vi polysaccharide vaccine is complicated by the living conditions (i.e., environment) of the study participants and the lack of knowledge regarding the exact microbial exposure (*S. typhi* or other infectious agents) of each study participant. It is desirable that similar studies be performed on participants residing in hygienic areas that lack natural exposure to *S. typhi*, and with access to good quality water and hygienic sewer services, although residents of such areas are unlikely to require vaccination for typhoid.

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