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Single Nucleotide Polymorphisms in the Toll-Like Receptor 3 and CD44 Genes Are Associated with Persistence of Vaccine-Induced Immunity to the Serogroup C Meningococcal Conjugate Vaccine

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The rate of decay of antibody concentration following serogroup C meningococcal (MenC) polysaccharide-protein conjugate vaccination varies between individuals. This depends partly on vaccination age but may be influenced by human genetics. We studied 721 single nucleotide polymorphisms (SNPs) across 131 candidate genes in a first cohort of 905 Caucasians (11 to 21 years old; mean time after vaccination, 4.9 years) and 30 SNPs across 17 genes in a replication study using 155 children, aged 6 to 12 years (mean time after vaccination, 6.7 years), and 196 infants (1 year old; mean time after vaccination, 8 months). Individuals were classified as responders or nonresponders for total MenC IgG concentration and MenC serum bactericidal antibody (SBA) measurements. Associated genes were examined further for quantitative outcome measures. Fifty-nine SNPs in 37 genes were associated with IgG persistence (adjusted for age at measurement), and 56 SNPs in 36 genes were associated with SBA persistence (adjusted for age at measurement and vaccine used). Three SNPs each within the Toll-like receptor 3 (TLR3) (rs3775291, rs3775292, and rs5743312) and CD44 (rs11033013, rs353644, and rs996076) genes were associated with IgG (adjusted for age at measurement) or SBA (adjusted for age at measurement and vaccine used) persistence in the initial genetic study (P, 0.02 to 0.04). Single SNPs within the TLR3 (rs7657186) ($P = 0.004$ [unadjusted]) and CD44 (rs12419062) ($P = 0.01$ [unadjusted]) genes were associated with IgG persistence in the replication study. These results suggest that genetic polymorphisms in the TLR3 and CD44 genes are associated with the persistence of the immune response to MenC vaccines 1 to 6 years after vaccination.

The United Kingdom was the first country to introduce an immunization program to control disease caused by serogroup C *Neisseria meningitidis*. At the time, it was the leading infectious cause of death in early childhood in the United Kingdom. Between 1999 and 2000, the serogroup C meningococcal (MenC) polysaccharide-protein conjugate vaccine was introduced for individuals under 18 years of age (later extended to those under 24 years of age) (4). The rate of vaccine uptake in the United Kingdom was >85% (4), with a vaccine efficacy estimate of 94% (22). Since 2000, only infants have been immunized with the MenC vaccine; rates of MenC disease have remained low due to individual protection from immunization and to herd immunity. For individuals vaccinated in early childhood, bactericidal antibody levels are waning rapidly, and population immunity may decline in the decade ahead (21).

The persistence of MenC vaccine immunity is dependent on the age of individuals at vaccination (19, 27). Vaccinated teenagers retain vaccine-induced antibody for 6 years, whereas protection in infants and toddlers declines rapidly (19). The immune response to vaccination is under genetic influence, with limited data suggesting that heritability ranges from 35 to 90%, depending on the vaccine (12). Differences in vaccine efficacy occur between different ethnic groups, suggesting a further role for genetic factors (12).

These studies are limited by small sample sizes and numbers of genetic markers, incomplete clinical and serological data, and difficulties in quantifying environmental factors. Vaccine-induced antibody levels as correlates of protection can be difficult to interpret, and arbitrarily chosen concentrations are used to define protection in populations.

The immunogenicity of meningococcal vaccines can be measured using the serum bactericidal antibody (SBA) titer or total specific IgG concentration. SBA assay measures functional antibodies and is considered a better correlate of protection (3). A titer of 1:4 or higher (with exogenous human complement) (9) or 1:8 or higher (with exogenous rabbit complement) is considered protective against serogroup C *Neisseria meningitidis* (2, 29). The total specific IgG concentration is measured using an enzyme-linked immunosorbent assay (ELISA) (8, 10).

We examined human genetic influences on the persistence of the immune response to MenC vaccines. In an initial genetic study ($n = 905$), we genotyped over 700 single nucleotide polymorphisms (SNPs) chosen on the basis of an *in vitro* expression study

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and a literature search. We used a second cohort ($n = 351$) to confirm the presence of associations.

MATERIALS AND METHODS

Study participants and vaccines. In the initial genetic study, volunteers in Buckinghamshire and Oxfordshire (United Kingdom) were recruited between March and December 2005 (26). Participants were healthy adolescents aged between 11 and 20 years who had received a primary MenC conjugate vaccine during the United Kingdom MenC vaccine campaign in 1999 to 2001. Exclusion criteria were described by Snape et al. (26).

Two groups of children were recruited for the replication cohort. The first group included children ($n = 155$) aged 6 to 12 years between September 2006 and July 2008, from Oxfordshire, United Kingdom (19). Participants received a primary MenC conjugate vaccine(s) in the 1999-2001 United Kingdom MenC vaccine campaign, approximately 6 years prior to sampling. Exclusion criteria were described by Perrett et al. (19).

The second group included 196 infants (aged 55 to 89 days) recruited to a phase II, open-label, randomized controlled trial between August 2004 and September 2006, from Oxfordshire, Buckinghamshire, and Berkshire, United Kingdom (27). Participants received a primary course of either a MenACYW-CRM_{197} conjugate vaccine (Menveo; Novartis Vaccines and Diagnostics, Siena, Italy), at 2 and 4 or 2, 3, and 4 months of age, or MenC vaccine (Menjugate; Novartis Vaccines and Diagnostics, Siena, Italy), at 2 and 4 months of age. The exclusion criteria were described by Snape et al. (27). Blood was obtained 8 months after the last priming dose and prior to boosting at a mean of 1.05 years (range, 361 to 425 days) after immunization.

After enrolment, previous MenC vaccination was confirmed using the participant's medical records or centralized immunization records, and 10 ml of whole blood was taken. For infants, the volume was 6 ml, taken at 12 months of age. The age at MenC vaccination was recorded, together with the exact vaccine given, when available.

Serological responses. A standardized ELISA was used to measure the MenC-specific IgG concentration (8) for the initial cohort and the first, but not second, group in the replication cohort. The cutoff for responders for the IgG ELISA was ≥ 2 μg/ml.

SBA titers for the initial genetic study and the first group in the replication cohort were measured at the Vaccine Evaluation Unit, Manchester, United Kingdom. Exogenous baby rabbit serum was used as the complement (rSBA) source. SBA titers for the second group of the replication cohort were measured at the laboratories of Novartis Vaccines, Marburg, Germany (8), using exogenous human complement (hSBA). The reference for all bactericidal assays was C11 (C:16:p1.7-1,1). The SBA titer was expressed as the reciprocal of the final serum dilution yielding 50% or greater killing after 60 min. The serologic cutoff for responders was an rSBA titer of $\geq$1:8 and an hSBA titer of $\geq$1:4. Participants with levels below 1:8 and 1:4, respectively, were termed nonresponders.

Genetic analysis. (i) DNA extraction. DNA extraction was performed using GeneCatcher gDNA blood kits (Invitrogen, United Kingdom) with whole blood collected in EDTA tubes. The extracted DNA was quantified using PicoGreen (Invitrogen, United Kingdom) and stored at -20°C.

(ii) *In vitro* expression study to identify candidate genes. Many genes involved in vaccine-induced immunity probably remain undescribed. We conducted an experiment with cells from a previously vaccinated Caucasian adult (aged 40 years) to discover unknown genes which may be potential candidates for vaccine-induced immunity. Peripheral blood mononuclear cells were cultured *in vitro* at a concentration of 2×10^5 cells per well with MenC vaccine (Menjugate), phytohemagglutinin (PHA; 5 μg/ml) (Sigma, United Kingdom), or medium alone for up to 2 weeks at 37°C. Differential expression of five genes (HPRT1, gamma interferon [IFN-γ], CD40 ligand [CD40L], interleukin-13 [IL-13], and NF-κB genes) was documented over the time course, using real-time PCR (Lightcycler; Roche, United Kingdom) (data not shown).

After demonstrating that the five genes were expressed differently between stimulated and unstimulated cells, the differential expression of mRNA between the vaccine-stimulated and unstimulated cells was examined across the human genome by use of Affymetrix chips (H-U133, version 2.0, chips; Affymetrix, United Kingdom). The log fold changes in gene expression were examined using the robust multichip average (RMA) and Microarray Suite (MAS 5.0) to normalize signals and identify differentially expressed genes. Linear models for microarray data (limma) and the *t* test statistic using limma were also used to determine the genes with differential expression. Each gene was ranked by differential expression, using the RMA log fold change, RMA *P* value, RMA limma *P* value, MAS 5.0 log fold change, and MAS 5.0 limma *P* value. Genes carrying SNPs associated with *P* values of <0.001 by at least three of the approaches were included in further analyses.

(iii) Candidate gene and SNP selection. Candidate genes for association with persistence of immune responses were selected using the *in vitro* expression study described above (in total, nine genes carrying 58 SNPs were selected; for further details, see below), and the remainder were chosen via a literature search.

The literature search was based on genes associated with vaccine-induced immunity (any vaccine), B or T cell immunity (any stimulus), or innate immune pathways. SNP selection was based on HapMap (http://www.hapmap.org/) frequency data, validation and anticipated genotyping success rate on the Illumina platform, and the distribution of SNPs across the locus. Coding changes were chosen preferentially, together with SNPs, in the sequence 500 bp into the 5′ and 3′ regulatory regions. Further selection was based on HapMap data on Yoruba (YRI) and/or individuals of European ancestry (CEU), and many of the SNPs were tag SNPs.

Genes containing SNPs associated with correlates of protection in the initial genetic study, with *P* values of ≤ 0.05, were candidates for inclusion in the replication study. Genes with more than one associated SNP were prioritized. SNPs found to be associated with the vaccine-induced antibody level in a parallel study on hepatitis B vaccine immunity were also included (11). In total, 710 SNPs in 131 genes were chosen using a literature search.

(iv) Genotyping. A custom Illumina Bead Array platform was made to examine the 768 SNPs in the initial genetic study. In the replication cohort, 34 SNPs were examined using the Sequenom platform (iPlex and hME).

(v) LD analysis. Linkage disequilibrium (LD) was evaluated in Haploview (http://www.broad.mit.edu/mpg/haploview/) using the default parameters, according to the method of Gabriel et al. (7).

Statistical analysis. (i) Initial genetic study. The presence of Hardy-Weinberg equilibrium (HWE) was examined for each SNP separately for IgG and rSBA by comparing responders and nonresponders, with the responders acting as the control group.

All of the data were analyzed using responders versus nonresponders as a binary outcome measure. Univariate associations with IgG and rSBA were examined for each SNP by using the chi-square or Fisher's exact test, as appropriate. Genotype data were analyzed as categorical variables, with 11 representing wild-type homozygotes, 12 representing heterozygotes, and 22 representing variant homozygotes. For polymorphisms with a minor allelic frequency (below 10 in absolute number), the heterozygotes and low-frequency homozygotes were grouped together.

Associations with nongenetic risk factors, including age at vaccination, gender, vaccine type, time between vaccination and measurement of immune responses to vaccination, and age at measurement, were analyzed by employing logistic regression, using IgG or rSBA (responders and nonresponders) as the dependent variable. All significant nongenetic factors were included in an adjusted analysis using separate models for each SNP. SNPs of interest were included in the replication study.

(ii) Replication study. HWE and adjusted associations for each SNP were examined as described for the initial genetic study. The genes with significant SNPs in the initial and replication studies were analyzed further for associations between phenotypic correlates of protection (i.e., IgG, rSBA, and hSBA) and genotype (as a categorical exposure variable). Separate logistic regression models, adjusted for nongenetic risk factors

(age at vaccination, gender, vaccine type or vaccine group, time between vaccination and measurement of immune responses to vaccination, and age at measurement), were run for each correlate (IgG, rSBA, and hSBA). Age at measurement was a significant factor associated with IgG. Age at measurement and vaccine type were shown to be significant factors associated with SBA. A meta-analysis containing the first cohort and the first group in the replication cohort was performed for all of the SNPs examined in the replication study, using the phenotypic correlate of protection and the genotype. The associated genes in the initial and replication cohorts were examined further using quantitative outcome measures. The IgG geometric mean concentrations (GMC) and the SBA geometric mean titers (GMT) were analyzed unadjusted for nongenetic risk factors, and the difference in genotype between the responders and nonresponders was compared using Student's *t* test.

Genetic associations were reported at the 5% significance level. Although with the large number of comparisons some of the associations may be significant based on chance alone, there is an argument against statistical adjustment for multiple testing (24). A weakness with Bonferroni's correction is that the interpretation of the finding depends on the number of tests performed and true significance can be lost due to the number of tests performed (18). Because of this issue, we took a more robust approach, assessing the possible signals in the first study by undertaking a replication study using two independent groups. The replication group contained two unrelated groups of individuals who were tested in a follow-up study. It is highly unlikely that the same gene would be significant in both replication groups, as well as the initial genetic study group, by chance, suggesting that the associated genes observed are in fact significantly associated. Therefore, interval estimates are reported where possible (i.e., odds ratios with confidence intervals), and exact *P* values are provided so that the reader may calculate a Bonferroni adjustment (alpha = 0.05/number of tests) (see Tables S1 and S2 in the supplemental material). A meta-analysis was performed using the initial cohort and the first group in the replication cohort for all of the SNPs studied in the replication cohort (see Table S3). Statistical analysis was performed using Stata software (version 10; Statacorp).

Ethical approval for the studies. Each substudy was granted approval by the Oxfordshire Research Ethics Committee, United Kingdom. The approval numbers were CO2.328 for the initial study, 06/Q1605/100 for the first group in the replication cohort, and 04/Q1604/28 for the second group in the replication cohort.

RESULTS

Study participants, vaccines, and serological responses in initial genetic study. In the initial genetic study, 999 individuals were recruited. Twenty-one non-Caucasians were excluded, and the DNAs from 73 participants failed to be genotyped. Therefore, 905 participants were analyzed. The ages at vaccination ranged from 6.5 to 16.4 years (mean, 10.08 years; interquartile range [IQR], 8.7 to 11.3 years); the ages at measurement ranged from 11 to 21 years (mean, 14.8 years; IQR, 13.1 to 16.3 years), with a mean of 4.9 years after priming with MenC vaccine (range, 3.8 to 6.3 years). There were 490 (54%) males (Table 1). Three different MenC vaccines were used: 619 (68.4%) participants received Menjugate, 80 (8.8%) participants received NeisVac-C (Baxter Vaccines, Beltsville, MD), and 83 (9.2%) participants received Meningitec (Wyeth, Pearl River, NY). The vaccine type was unknown for 123 (13.6%) participants. GMC results for IgG were available for 897/905 individuals (99%), and GMT results for rSBA were available for 869/905 individuals (96%).

Study participants, vaccines, and serological responses in replication study. The replication cohort contained 351 participants comprising two patient groups, which were analyzed separately. The first replication group included 250 children: for 163 of these children, samples were available for the genetic study, but there was insufficient blood available for 87 children. Seven of the children providing blood samples were non-Caucasian and were excluded, and 1 failed genotyping, leaving 155 participants for analysis (Table 1); 86 (55%) of these were male. Blood sampling was performed a mean of 6.75 years (range, 5.94 to 7.25 years) after MenC vaccination. The mean age at vaccination was 2.6 years (range, 0.2 to 6.5 years; IQR, 1.0 to 4.2 years), and the mean age at measurement was 9.2 years (range, 6.4 to 12.6 years; IQR, 7.8 to 10.9 years). The vaccines given were Meningitec for 139 (89.7%) participants, Menjugate for 5 (3.2%) subjects, and NeisVac-C for 1 (0.6%) person. For 10 participants (6.5%), the MenC vaccine used was unknown.

TABLE 1 Age and sex distribution by vaccine response measure (IgG and SBA) for the initial and replication studies

Study and group[a]	No. of participants	Mean age (yr)	No. (%) of males
Initial genetic study			
IgG responders	472	15.30	267 (56.60)
IgG nonresponders	425	14.40	219 (51.50)
rSBA responders	728	14.90	76 (53.90)
rSBA nonresponders	141	14.30	397 (54.50)
Replication study			
School-aged children			
IgG responders	7	11.60	3 (42.90)
IgG nonresponders	148	9.10	83 (56.10)
rSBA responders	37	9.80	18 (48.60)
rSBA nonresponders	118	9.00	68 (57.60)
Infants			
hSBA responders	17	1.05	43 (43.00)
hSBA nonresponders	179	1.05	53 (55.00)

[a] IgG responders were participants with IgG concentrations of ≥2 μg/ml, and nonresponders were participants with IgG concentrations of <2 μg/ml. rSBA responders were participants with rSBA response levels of ≥1:8, and nonresponders were participants with rSBA response levels of <1:8. hSBA responders were participants with hSBA response levels of ≥1:4, and nonresponders were subjects with hSBA response levels of <1:4.

The second replication group included 225 individuals, with 217 available for genetic analysis; 16 were non-Caucasian and were excluded, 3 individuals failed genotyping, and 2 individuals did not have an hSBA result, leaving 196 infants (aged 55 to 89 days) (Table 1). The mean age at enrolment was 1.1 years (range, 1.1 to 1.2 years; IQR, 1.02 to 1.07 years), with blood taken 8 months later and prior to boosting. Ninety-six (49%) participants were male (Table 1). One hundred sixty-four (84%) participants received the MenACYW vaccine, and 32 (16%) participants received the MenC vaccine (Menjugate). The hSBA level was available for all 196 participants.

The GMC for IgG in the initial genetic study ($n = 897$) was 2.03 μg/ml (95% confidence interval [95% CI], 1.8 to 2.3 μg/ml), and that for the first replication group ($n = 155$) was 0.26 μg/ml (95% CI, 0.2 to 0.3 μg/ml). The GMT for rSBA in the initial genetic study ($n = 869$) was 227.31 (95% CI, 192.6 to 268.3), and that for the first replication group ($n = 155$) was 5.0 (95% CI, 3.8 to 6.6); the GMT for hSBA in the second replication group ($n = 196$) was 6.81 (95% CI, 6.0 to 8.3).

Whole-genome *in vitro* expression study. The *in vitro* expression study using Affymetrix chips revealed nine genes (CD163, CD58, LGMN, RAPH1, RANGAP1, RASSF5, TAPBP, ZAP70, and

GPNMB genes [see Table S4 in the supplemental material]) with differential expression by at least three statistical tests. These were thus included as candidate genes in the association study.

Genetic analysis of initial genetic study. Among 768 candidate SNPs genotyped, 33 SNPs had call rates of <90% and 14 SNPs were monomorphic or had a minor allele frequency (MAF) of ≤0.01 and were excluded (see Table S5 in the supplemental material). The remaining 721 SNPs were distributed across 131 genes (see Table S1). SNPs were distributed across most chromosomes (excluding chromosomes 15 and 18). The average genotyping success rate was 99.3% (range, 76.1 to 100.0%).

Of the 721 SNPs available, 49 (6.8%) and 58 (8.0%) SNPs in the responder group diverged from HWE for IgG and rSBA, respectively (P values ranged from <0.001 to 0.048). There was no clustering of these SNPs by locus.

Genetic analysis of replication study. Seven genes with more than one associated SNP were examined in the replication study together with SNPs associated in a parallel study (11). Out of 34 SNPs chosen, 4 SNPs (11.8%) failed to be genotyped in any sample (see Table S2 in the supplemental material), leaving 30 SNPs for analysis. In the first replication group, five (16.7%) SNPs in the responder group diverged from HWE for IgG, and a single SNP (3.3%) diverged from HWE for SBA. There was no clustering of these SNPs by locus. In the second replication group, two SNPs (6.7%) in the responder group diverged from HWE.

Statistical analysis. (i) Nongenetic risk factors associated with IgG and SBA levels in initial genetic study. Age at vaccination and age at measurement were positively associated with the IgG response. These variables were very strongly correlated and could not be included in the model due to colinearity. Age at measurement was the better predictor, as identified using the likelihood ratio test ($P < 0.0001$), and was included in the adjusted model. Sex and vaccine type did not affect the IgG response and were thus not adjusted for.

For rSBA, type of vaccine used ($P = 0.007$) and age at measurement ($P < 0.0001$), but not sex or age at vaccination, were significant nongenetic risk factors and were included as covariates in the analysis.

(ii) Nongenetic risk factors associated with IgG and SBA levels in replication study. Age at measurement was positively associated with the mean IgG concentration and SBA titer in the first group of the replication cohort ($P = 0.0002$ for IgG and $P = 0.02$ for SBA); all other nongenetic variables were not significant. The vaccine group was not associated with SBA, due to the small number of nonresponders across the groups. There was no significant association between SBA and age at measurement for the second group of the replication cohort ($P = 0.66$); vaccine type was significantly associated with SBA in the second group ($P < 0.0001$).

One explanation for the difference in significance between the initial genetic study and the first group of the replication cohort is that the children who were vaccinated at a younger age were measured for an SBA response earlier than the older children (with younger children measured at a mean of 4.7 years postimmunization and older children measured at a mean of 5.3 years postimmunization).

(iii) Genetic risk factors associated with IgG and SBA levels in initial genetic study. An unadjusted statistical analysis showed an association of 64 SNPs in 39 genes with the IgG (31 SNPs in 25 genes) or SBA (35 SNPs in 19 genes) level, with P values of <0.05, in the initial genetic study. Five genes were associated with both IgG and SBA (see Table S6i in the supplemental material). After an age at measurement-adjusted analysis, 59 SNPs from 30 genes were associated with the IgG level (P value range, 0.003 to 0.05). Fifty-six SNPs in 30 genes were associated with the rSBA level after adjusting for age at measurement and vaccine type (P value range, 0.001 to 0.05) (see Table S6ii). For the nine genes included based on the *in vitro* data and the Affymetrix chip analysis, five genes (LGMN, RAPH1, RASSF5, ZAP70, and GPNMB genes) (see Table S4) were significantly associated unadjusted. These SNPs were not associated after adjusting for age at measurement for IgG (and for vaccine type for SBA).

Overall, the Toll-like receptor 3 (TLR3) gene was the most significant gene associated with IgG and SBA. Further associated genes in the initial genetic study ($P < 0.005$) which were chosen for the replication cohort were the RAPH1, IL-10, IL-4, IL-7, CD44, and ITGAL genes. Eight additional genes were chosen based on significance in a parallel genetic study (11) (IL28RA, TMEM 37, ICOS, TAP2, CD163, CD58, MAPK8, and IFN-γ genes).

(iv) Genetic risk factors associated with IgG and SBA levels in the replication cohort. Two of the associated genes in the initial cohort were also associated with the IgG response in the replication cohort (but not with the SBA level). These were the TLR3 (rs7657186) ($P = 0.004$, unadjusted) and CD44 (rs12419062) ($P = 0.01$, unadjusted) genes. A meta-analysis containing the first cohort and the first group in the second cohort showed a significant association for 20 of the 30 SNPs examined (P values of <0.0001 to 0.001 [see Table S3 in the supplemental material]).

Because of the association of the TLR3 and CD44 genes, the genetic associations between the responders and nonresponders were studied further by examining the quantitative data, using GMC for IgG (unadjusted) and GMT for SBA (unadjusted) (see Table 3).

Genetic risk factors associated with IgG and SBA levels in both studies. (i) TLR3 gene. Three SNPs within the TLR3 gene were associated with the responder and nonresponder groups in the initial genetic study (rs5743312, rs3775291, and rs3775292) (Table 2). A different SNP in TLR3 was associated in the replication study (rs7657186) (Table 2). All four SNPs were associated in the meta-analysis (P values of <0.0001 to 0.001) (see Table S3 in the supplemental material). A single SNP in TLR3 was out of HWE (rs7657186) ($P = 0.001$ for the responder group and IgG in both cohorts). Variable LD exists between these SNPs, as shown in Fig. 1.

When the quantitative outcome was studied, the geometric mean IgG concentration was significantly lower for individuals heterozygous for the rs5743312 SNP than for those with the wild-type homozygous genotype (mean difference = 0.64; $P = 0.02$). The IgG concentration was lower, but not significantly, for the homozygous variant alleles with rs5743312, rs3775292, and rs7657186 compared with the wild-type homozygous genotype ($P = 0.07$, $P = 0.21$, and $P = 0.1$, respectively) (Table 3). SBA levels for the rs7657186 SNP were significantly lower for the mutant homozygous genotype than for the wild-type homozygous genotype (mean difference = 140.5; $P = 0.04$). This was not observed in the replication study (data not shown), probably because of the smaller numbers of responders.

(ii) CD44 gene. Overall, four SNPs in the CD44 gene were significantly associated with IgG or SBA. Two SNPs were associated in the initial cohort: rs11033013 ($P = 0.03$) with IgG and

TABLE 2 SNPs examined in the TLR3 and CD44 genes in the initial and replication studies

Gene and SNP	*P* value[a]									
	Initial genetic study				Replication study					
					School-aged children				Infants	
	IgG[b]		SBA[c]		IgG[b]		SBA[c]		SBA[d]	
	12	22	12	22	12	22	12	22	12	22
TLR3										
rs7657186	0.52	0.07	0.54	0.08	0.002*	**	0.20	0.76	0.29	0.93
rs5743312	0.07	**0.05**	0.67	**0.04**	1.00*	1.00	0.88*	1.00*	0.91	0.46
rs3775292	0.68	0.03	0.93	0.48	0.14*	1.00*	0.82	0.64	1.00	0.80
rs3775291	0.03	0.56	0.37	0.88	0.14*	1.00*	0.31	0.94	0.99	0.77
CD44										
rs353644	0.16	0.88	**0.02**	0.50	1.00	1.00	0.57	0.07	0.36	0.28
rs11033013	**0.03**	**0.03**	0.65	1.00						
rs4756196	0.56	0.64	**0.05**	0.35						
rs12419062	0.38	0.49	0.36	0.50	0.01*	**	0.76	0.19	0.19	0.21

[a] Adjusted logistic regression models were used. The *P* values shown are for genotype 12 (heterozygous variant) or 22 (homozygous variant) compared to the homozygous wild-type genotype (11 = baseline) for IgG or SBA (with significant values shown in bold). Subjects were separated into responders (IgG level of ≥2 μg/ml or SBA level of ≥1:8) and nonresponders (IgG level of <2 μg/ml or SBA level of <1:8) for analysis. The initial genetic study contained 905 individuals, and the replication cohort comprised school-aged children ($n = 155$) and infants ($n = 196$). Gray shading denotes SNPs that were not examined in the replication cohort. *, Fisher's exact *P* value is reported because no responders were observed; **, no wild-type or variant homozygous nonresponders were observed.

[b] With adjustment for age at measurement.

[c] With adjustment for age at measurement and vaccine type.

[d] With adjustment for vaccine type.

rs353644 ($P = 0.02$) with SBA. A different SNP was associated with IgG concentration (rs12419062) ($P = 0.01$) in the replication study (Table 2). One SNP in CD44 was out of HWE (rs12419062) ($P < 0.0001$ for responder group and IgG in the replication cohort). Only rs12419062 remained significant in a meta-analysis ($P < 0.0001$ for IgG and SBA).

When the quantitative outcome was examined, the geometric mean concentration of IgG was significantly higher for individuals with the homozygous variant allele of rs11033013 (mean difference = 1.24 μg/ml; $P = 0.04$) and for subjects heterozygous for rs996076 (mean difference = 0.72 μg/ml; $P = 0.03$), as shown in Table 3. There was a nonsignificant dose effect for the variant allele with rs12419062, with the lowest IgG level for individuals homozygous for the variant allele.

DISCUSSION

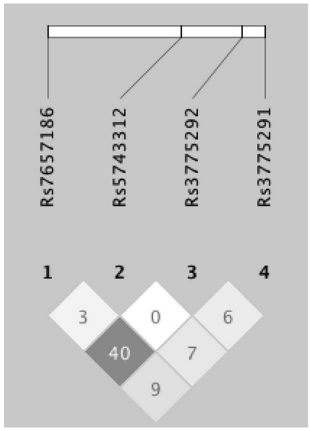
We have examined human genetic influences on the immune response to MenC vaccines in Caucasian children in the United Kingdom and found significant associations between polymorphisms in the TLR3 and CD44 genes and the persistence of immune responses to a MenC conjugate polysaccharide vaccine. There is considerable individual variation in the immune protection produced in response to vaccines for both viral and bacterial infections; it is reasonable to expect genetic factors to play a role in this variation, given the known heritability of vaccine responses (15, 20) and reports on positive candidate gene association studies (20, 25). The HLA region has been examined extensively for associations, and increasingly, non-HLA genes, such as those assessed here, have been associated with vaccine-induced immune responses.

There are a limited number of studies examining vaccine-induced immunity to Gram-negative bacteria. One study recently attributed some of the *Haemophilus influenzae* type b (Hib) vaccine efficacy to genetic factors (13). A further study examining the genetic determinants of the immune response to a pure polysaccharide vaccine for typhoid described (among others) an association with polymorphisms in the TLR1 and CD44 genes (14). Similar immune responses may be induced by the different vaccines for infections with Gram-negative organisms, as all contain polysaccharides from Gram-negative bacteria. Two studies describing vaccine-induced immunity to the plain polysaccharide MenC vaccine found a correlation between antibody concentrations and IgG allotypes (1, 17).

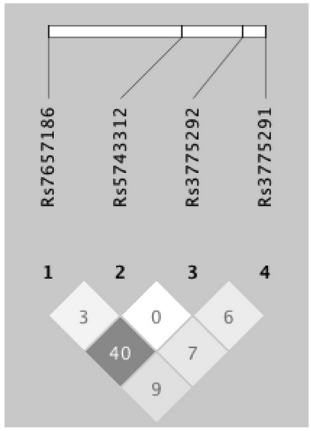
In the current study, SNPs in two genes (TLR3 and CD44 genes) were associated with vaccine-induced immunity to MenC in both an initial study and a replication study. Two measures of immunogenicity in response to the MenC vaccine were examined (IgG and SBA levels). The immune response measurements for the two outcomes are different, as the IgG level measures total specific antibody in an ELISA which detects high-avidity antibodies but may also detect lower-avidity antibodies and the SBA level measures a subset of IgGs (mainly IgG1) which have high avidity, are functional, and are able to kill bacteria in the presence of exogenous complement. Therefore, it is highly plausible that a SNP would be associated significantly with one measurement and not with the other. Three SNPs in the TLR3 gene were associated with these measures in the first cohort, and a different SNP was associated in the second cohort. A meta-analysis identified all four SNPs associated with IgG and SBA. When these SNPs were examined for quantitative outcomes, the presence of the variant allele appeared to decrease the GMC for IgG for two SNPs (rs5743312 and rs3775292), and the heterozygous form of two SNPs showed the highest GMC (rs3775291 and rs7657186). We suggest that these may influence the amount of antibody produced and could be relevant to the immune response after vaccination. A previous study found that individuals heterozygous for TLR3 SNP

TLR3

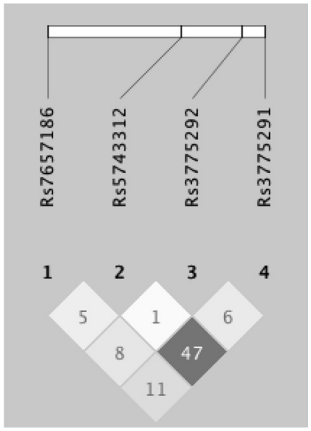
a)



b)

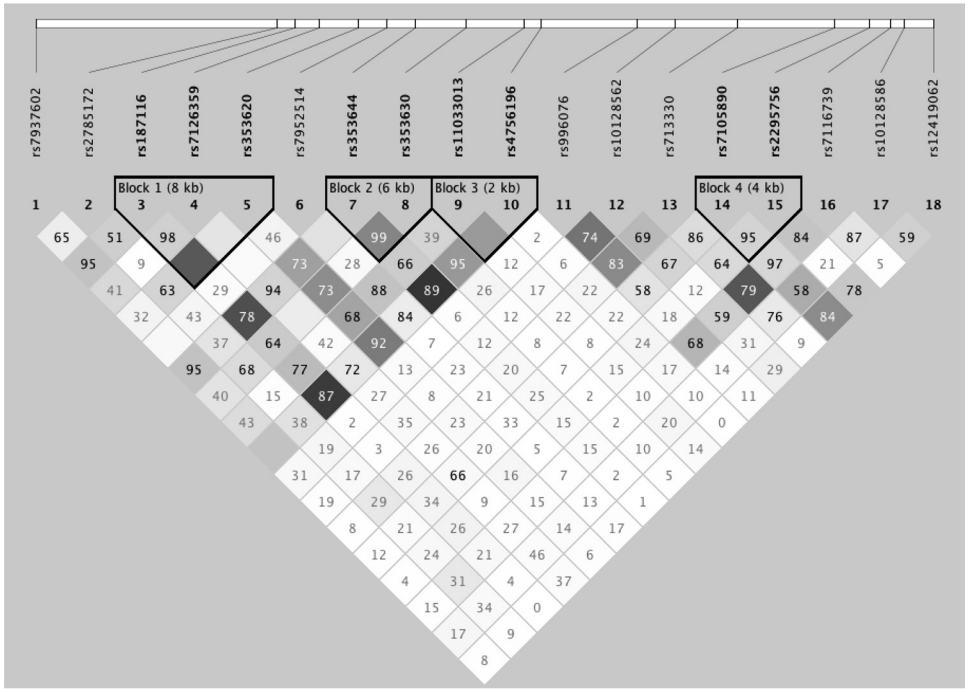


c)



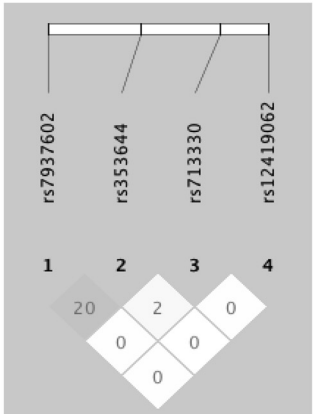
CD44

d)



CD44

e)



f)

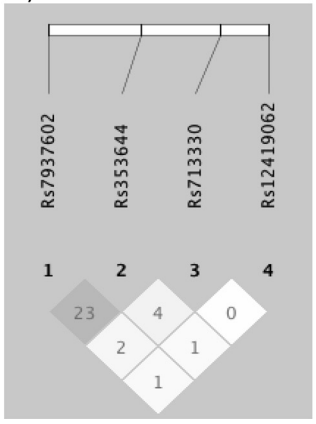


TABLE 3 GMC of IgG and GMT of SBA by genotype for TLR3 and CD44 gene SNPs in the initial genetic study[a]

Gene and SNP_genotype[b]	IgG GMC	95% CI	No. of individuals with genotype	*P* value[c]	SBA GMT	95% CI	No. of individuals with genotype	*P* value[c]
TLR3								
rs5743312_11	2.24	1.94–2.59	653	ref	244.78	201.48–297.37	634	
rs5743312_12	1.60	1.27–2.02	223	0.02	172.75	123.24–242.15	215	0.08
rs5743312_22	1.06	0.52–2.15	21	0.07	415.87	193.14–895.46	20	0.35
rs3775291_11	1.87	1.57–2.23	425	ref	201.38	157.6–257.32	413	ref
rs3775291_12	2.20	1.83–2.64	395	0.21	242.37	189.34–310.25	380	0.30
rs3775291_22	2.06	1.33–3.2	76	0.67	325.53	186.37–568.6	75	0.13
rs3775292_11	2.09	1.79–2.44	565	ref	231.53	188.19–284.85	545	ref
rs3775292_12	2.00	1.63–2.44	303	0.74	234.27	175.72–312.33	297	0.95
rs3775292_22	1.33	0.60–2.94	29	0.21	112.58	39.79–318.53	27	0.14
rs7657186_11	1.98	1.69–2.32	551	ref	236.17	192.31–290.03	533	ref
rs7657186_12	2.23	1.83–2.71	315	0.37	233.15	174.12–312.19	304	0.94
rs7657186_22	1.11	0.53–2.33	30	0.10	95.71	35.69–256.66	31	0.04
CD44								
rs353644_11	2.14	1.68–2.72	230	ref	264.19	191.13–365.19	220	ref
rs353644_12	1.84	1.55–2.19	451	0.13	189.99	150.37–240.06	437	0.11
rs353644_22	2.32	1.82–2.96	215	0.63	288.14	205.06–404.88	211	0.72
rs11033013_11	1.86	1.57–2.21	488	ref	221.94	177.79–277.06	471	ref
rs11033013_12	2.11	1.74–2.54	339	0.35	218.9	166.87–287.13	332	0.94
rs11033013_22	3.10	2.04–4.72	69	0.04	313.5	163.27–601.97	65	0.29
rs996076_11	1.68	1.31–2.14	246	ref	234.08	170.77–320.85	240	ref
rs996076_12	2.40	2.00–2.87	440	0.03	250.58	196.97–318.79	421	0.12
rs996076_22	1.76	1.42–2.19	211	0.76	180.42	129.22–251.89	208	0.26
rs12419062_11	2.37	1.68–2.35	378		245.99	190.9–316.90	365	
rs12419062_12	1.93	1.61–2.31	411	0.66	202.84	158.38–259.77	402	0.29
rs12419062_22	0.38	0.23–0.52	107	0.45	272.31	168.12–441.08	101	0.71

[a] The samples were analyzed using Student's *t* test with the geometric mean concentration or geometric mean titer as a continuous variable, without adjustment.
[b] 11, wild-type homozygous genotype; 12, heterozygous genotype; 22, variant homozygous genotype.
[c] ref, referent/baseline group.

rs3775291 had a lower immune response to a measles vaccine (5); this was also observed in responders in this study (with IgG responses of ≥2 μg/ml). Seven SNPs within the TLR3 gene have been described to be associated with vaccine-induced immunity to viral vaccines (rs6822014, rs11721827, rs13126816, rs3775296, rs5743305, rs1914926, and rs3775291) (5, 16). The current work increases the number of SNPs in the TLR3 gene affecting vaccine responses to 10.

TLRs are important mediators of immunity, as human pathogen recognition receptors identifying specific ligands, such as bacteria, viruses, or fungi (K. Takeda and S. Akira, presented at the Seminars in Immunology, 2004), triggering the innate immune responses and inducing the adaptive immune response. TLR3 recognizes double-stranded RNA (dsRNA) though the N-terminal ectodomains; the bound form triggers the MyD88-independent signaling cascade and activates the NF-κB and interferon regulatory factor (IRF) transcription factors. These transcription factors induce the expression of inflammatory cytokines, type I interferons, and chemokines.

Whereas it is possible that TLR3 has a broader involvement in the host response than recognizing viral dsRNA, there are few reports which examine the association of TLR3 with other ligands. One study reported upregulation of TLR3 in granulocytes and monocytes in response to Gram-positive and -negative bacteria (*Staphylococcus aureus* and *Escherichia coli*) (28). SNPs within the TLR3 gene may influence the immune response to vaccination against bacterial diseases such as MenC (28). Two possible explanations are that MenC vaccines could directly trigger TLR3, as they may contain dsRNA, and that injection of the vaccine may induce release of an endogenous dsRNA which activates TLR3.

We found four SNPs in the CD44 gene associated with MenC vaccine-induced immunity. Two SNPs in the initial cohort and a different one in the replication cohort were associated with IgG or SBA in the responder and nonresponder groups. A single SNP (rs12419062) was significant in the meta-analysis. Two of the SNPs were associated with IgG GMC and SBA GMT when the quantitative measures were examined, with the heterozygous vari-

FIG 1 Graphical representation of LD across the TLR3 gene and the CD44 gene in the initial and replication studies. Plots were generated using Haploview (http://www.broad.mit.edu/mpg/haploview/) with default parameters, as defined by Gabriel et al. (7). Pairwise LD is shown by the r^2 values indicated in the squares, with the numbers reporting the r^2 values multiplied by 100 (ranging from 0 to 100). The values are denoted in white ($r^2 = 0$), gray ($0 < r^2 < 1$), or black ($r^2 = 1$) squares. The name and relative position in the gene of each SNP are indicated above the plot. TLR3 data are shown for the initial study (a), the first group in the replication cohort (b), and the second group in the replication cohort (c), based on genotype data for 4 SNPs. CD44 data are shown for the initial study (based on genotype data from 18 SNPs), indicating the presence of 4 small LD blocks within our set of markers (d) or depicting only the 4 SNPs also examined in the replication cohort (e), and for the first group in the replication cohort, using genotype data from 4 SNPs (f).

ant of two SNPs (rs11033013 and rs996076) associated with the highest IgG level and the variant allele of rs12419062 nonsignificantly associated with a decrease in IgG GMC. A parallel study implicated CD44 variants in immunity induced by hepatitis B vaccination (11, 23). Although there are differences between the studies (use of a viral vaccine and different ethnic populations), this may indicate commonalities in the mediation of responses to viral and bacterial vaccines via CD44.

CD44 is involved in leukocyte adhesion and transendothelial migration. *N. meningitidis* strains adhere to endothelial cells and interfere with leukocyte transmigration (6). Membrane integral proteins such as CD44 are recruited to host complexes containing bacteria (6), and diapedesis of the leukocytes stops, rendering the host defenseless. CD44 thus has a role in the host response to the MenC vaccine.

The current study is based on a large, random, ethnically homogenous sample, meaning that any confounding effects due to population stratification are unlikely. The data were spread over a range of children's ages. A limitation is that the replication cohort contained smaller numbers of participants and that SBA but not IgG was examined in one of the replication groups. As previously described (19), the number of participants with an IgG concentration of ≥ 2 μg/ml was small in the first replication group. This may be because the study group was younger and vaccine-induced immune persistence is shorter in younger children. The ages of the replication cohorts do not overlap, and it is therefore difficult to compare the two groups. The positive findings will not be affected, but it is possible that genetic factors specific to age at vaccination may have been missed. A limited number of the associations in the initial genetic study were followed up in the replication study, but some of those not examined may have been positive in the replication study. The meta-analysis of the initial genetic study and the first group in the replication study underscored the significance of TLR3 and suggested that rs7937602 was the most significant SNP within the CD44 gene. Another 14 SNPs were significant in the meta-analysis of both studies, and it would be interesting to examine these further.

This is the first report to find associations between polymorphisms in the TLR3 and CD44 genes and the persistence of immune responses to a MenC conjugate polysaccharide vaccine. Further studies to investigate the role of these genes in immunity may allow the design of vaccines to improve persistence of antibody, thereby improving immunity to MenC infection.

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