Typhoid fever : aspects of environment, host and pathogen interaction

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Polymorphisms in pro-inflammatory genes and susceptibility to typhoid and paratyphoid fever

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4 Abstract

Host genetic factors are thought to contribute to susceptibility and outcome in infectious diseases. A polymorphism in a pro-inflammatory gene, tumor necrosis factor alpha (*TNFA–308*), was recently found to be associated with susceptibility to typhoid fever. Polymorphisms in other pro-inflammatory genes may also contribute to susceptibility to typhoid fever. We tested this hypothesis in a case-control study in an endemic area in Indonesia. Samples of patients with blood culture-confirmed typhoid fever (n=90) and paratyphoid fever (n=26), and fever controls (n=337), were compared with those of community controls (n=322). In these groups we analyzed polymorphisms in *TNFA* by PCR and RFLP, polymorphisms of *IFNG*, *IL1A*, *IL1B*, *IL1R1*, *TNFRSF1A*, *CASP1* and *CRP* by Sequenom MassArray, and polymorphisms in *IL12B* and *IFNGR1* by fragment length analysis. The *IL1R1* polymorphisms were nearly absent in the Indonesian population. The *TNFA–308* polymorphism was not associated with typhoid fever (OR 0.35 [95%-CI: 0.1-1.0]) in this population. The polymorphisms at *TNFA–238* or in *IFNG*, *IL1A*, *IL1B*, *IL12B*, *TNFRSF1A*, *IFNGR1*, *CASP1* and *CRP* were also not associated with typhoid or paratyphoid fever. We conclude that polymorphisms in pro-inflammatory genes appear not to contribute to susceptibility to typhoid fever and, in view of earlier findings, suggest that the *TNFA–308* polymorphism might be related to severity of established disease rather than to susceptibility per se.

Introduction

Typhoid fever, caused by *Salmonella typhi* and transmitted by fecal-oral route through contaminated food, water and drinks, is a health concern in many developing countries such as Indonesia [1]. Paratyphoid fever, caused by *Salmonella paratyphi A*, *B* or *C*, has a disease presentation highly similar to that of typhoid fever, but – at least in Jakarta – seems to follow a distinct route of transmission: whereas typhoid fever is spread predominantly within the household, paratyphoid fever is mainly transmitted outside the patient's home [2]. The identification of risk factors and most relevant route of transmission of enteric fever (encompassing both typhoid and paratyphoid fever) are essential for the development of control strategies and allocation of public health resources.

Salmonellae are able to survive and multiply within mononuclear phagocytes of lymphoid follicles, liver and spleen [3]. In reaction to the presence of this facultative intracellular pathogen, the host mounts an immune response. The development of the systemic and local immune response is a complex balancing act, in the course of which host immunological mediators as well as bacterial factors may contribute to tissue damage such as the necrosis of Peyer's patches and thereby complications in severe typhoid fever, i.e., bleeding and bowel perforation [4].

Unlike for some other infectious diseases caused by an intracellular pathogen such as tuberculosis and leprosy, no historical data is available suggesting typhoid fever would have a genetic component. Recently however, an association between single nucleotide polymorphism (SNP) *TNFA* –308 and typhoid fever has been reported in Vietnam. Together with HLA-DRB1*0301/6/8 and HLA-DQB1*0201-3, the *TNFA–308*A* allele was thought to be associated with susceptibility to typhoid fever [5]. No association of a second common polymorphism, i.e., *TNFA–238*, and susceptibility to typhoid fever was found [5]. Similar to *TNFA-308*, polymorphisms in and around other genes encoding pro-inflammatory cytokines and their receptors, may also contribute to an individual's susceptibility to enteric fever. Besides Tumor Necrosis Factor-α (TNF-α), also Interferon-γ (IFN-γ), Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), Interleukin-12p40 (IL-12p40), the receptors Tumor Necrosis Factor Receptor 1 (TNFR1), Interferon-γ receptor 1 (IFN-γR1), and IL-1 receptor (IL-1R1), and the IL-1β-activating Caspase-1 (CASP1) and the inflammatory biomarker C-reactive protein (CRP) are involved in many of the cellular and inflammatory processes underlying the immune reaction. Indeed, the *IFNG* SNP +874 influences IFN-γ production [6] and has been found in various studies to be associated with susceptibility to tuberculosis [6-8]. An allele of the CA repeat polymorphism in intron 5 of *IFNGR1* is associated with susceptibility to tuberculosis [9]. In the IL-1 complex *IL1A* SNP -889 in the promoter is associated with juvenile rheumatoid arthritis [10]. *IL1B* SNP -511 in the promoter is associated with amongst others EBV seronegativity [11] and homozygosity of this marker is associated with mortality

from meningococcal disease [12]. *IL1B* SNP +3953 in exon 5 influences IL-1 β expression levels [13] and is associated with several inflammatory disorders. The *IL1R1* SNPs A124G and R456R are both in the coding region and have not been studied extensively yet. Associations between polymorphisms in *CASP1* (also known as *ICE*) and disease have not been studied in humans so far, a polymorphism in *Casp1* has however been found to influence salmonella disease resistance in poultry [14]. The human *CASP1* SNP in codon 235 has been extensively validated and has a high percentage of heterozygosity (25%, source: SNP database). The *IL12B* insertion/deletion polymorphism in the promoter has been reported to influence both *IL12B* mRNA expression levels [15] and, as a result, nitric oxide (NO) production [16], this polymorphism is in addition associated with a.o. mortality from cerebral malaria [16] and outcome of hepatitis C virus infection [17]. *TNFRSF1A* SNP +36 is associated with Crohn's disease [18] and familial rheumatoid arthritis [19]. *CRP* SNP +1444 influences CRP production [20] while serum CRP levels were found to be a marker for infection with *Salmonella typhi* [21].

In the present study, to determine whether polymorphisms of *TNFA* and other pro-inflammatory genes are associated with an individual's susceptibility to typhoid fever and paratyphoid fever we assessed the prevalence of the polymorphic alleles in patients enrolled in a community based surveillance. To this end we analyzed the above described polymorphisms in the genes *TNFA*, *IFNG*, *IL1A*, *IL1B*, *IL12B*, *TNFRSF1A*, *IFNGR1*, *IL1R1*, *CASP1* and *CRP* and studied the association of these polymorphisms in a typhoid fever case-control study.

Materials and Methods

Study design and participants

From June 2001 to February 2003 patients with bloodculture-confirmed *Salmonella typhi* (n=69) or *Salmonella paratyphi A* (n=24) were identified in a prospective, community-based, passive surveillance study in the Jatinegara district of Jakarta, Indonesia [2]. Participants were 1019 consecutive individuals living in the study area who presented with fever lasting 3 days or more to one of 24 healthcare facilities in the district. Blood cultures were collected into Bactec bottles (aerobic) containing antibiotic absorbing resins (Becton Dickinson, USA) that were provided to the centres by the study group free of charge. Every second consecutive non-enteric fever patient was selected as a fever control. Also, during the surveillance community controls were randomly selected within a random household in every third *rukun tetangga* (RT) of a total of 1140 RTs in the study area, RT being the smallest administrative unit of 40-60 households. When a community control reported fever in the 30 days preceding the interview or refused participation, the house on alternating sides of the initially selected household was approached. The selection of both groups

of controls was non-matched for age, sex or neighbourhood to limit selection-bias and prevent overmatching. Four controls from both groups for every case of enteric fever were selected in order to increase statistical power. Full details of the enrollment of patients have been described in detail elsewhere [2]. Furthermore, between March and October 2003, 4 participating centers and the Medistra Hospital adjacent to the study area contributed another 23 cases of enteric fever (i.e., 21 typhoid fever and 2 paratyphoid fever cases). All cases and fever controls were visited at home within one month after the febrile episode that led to the blood culture. Community controls were visited randomly throughout the study period. This study was approved by the Indonesian National Institute of Health Research and Development (*Litbangkes*) and provincial authorities. From all participants or their guardians a written informed consent was obtained.

Household visits and sample collection

Typhoid and paratyphoid cases, fever controls and randomly selected community controls were interviewed by trained medical school graduates using a validated, standardized questionnaire as described [2,22]. Blood was collected using an EDTA-containing vacutainer system (Becton Dickinson, USA). Genomic DNA from white blood cells was isolated essentially as described by Sambrook [23]. The isolated DNA was dissolved in 10 mM Tris, 0.1 mM EDTA, pH 7.5.

TNFA Single Nucleotide Polymorphism (SNP) genotyping

Determination of *TNFA–238* and *TNFA–308* SNPs was done using PCR amplification followed by restriction enzyme digestion as described [24]. Samples were run on EL300 Spreadex gels in a SEA 2000 electrophoresis apparatus (Elchrom Scientific AG, Cham, Switzerland). Interpretation of gels demonstrating the *TNFA–238* and *TNFA–308* SNPs alleles was done twice in a blinded fashion and independently by two laboratory workers.

Sequenom typing of single nucleotide polymorphisms in pro-inflammatory genes

SNPs in the genes *IFNG*, *IL1A*, *IL1B*, *TNFRSF1A*, *IL1R1*, *CASP1* and *CRP* were selected from literature and databases on the web (**Table 2**). Multiplex assays were designed using Assay designer software (Sequenom). Genotyping was performed using the MassArray platform according to manufacturers protocols (Sequenom). In brief, after PCR on 2.5 ng of DNA a primer extension reaction was performed to introduce mass-differences between alleles and, after removing salts by adding a resin, ~15 nl of the product was spotted onto a target chip with 384 patches containing matrix. Mass differences were detected using a Bruker Autoflex MALDI-TOF mass spectrometer and genotypes were assigned real-time using Typer 3.1 software (Sequenom). As quality control, 10% of samples were genotyped in duplo and no inconsistencies were observed. Primer sequences are available upon request.

Analysis of IFNGR1 CA repeat and IL12B ins/del polymorphisms

PCRs for *IFNGR1* CA and *IL12B* ins/del polymorphisms were performed using 100 ng of genomic DNA, 200 μM of each dNTP, 10 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase (Promega) in a total volume of 25 μl. Forward primers were 5'-labeled with FAM and TET respectively, primer sequences are available on request. PCR conditions used: 5 min 95˚C, followed by 30 cycles of 95˚C 30 s, 55˚C 30 s, 72˚C 30 s and 5 min 72˚C. PCR products were diluted 1:20 in H_2O, 1 μl of diluted product was added to 8.8 μl HiDi Formamide, 0.2 μl 400 HD-ROX size standard (Applied Biosystems) and heated to 95˚C for 5 min. Products were run on an ABI Prism 3700 DNA Analyzer (Applied Biosystems), results were analysed using GeneScan Analysis and Genotyper software (Applied Biosystems). Several homozygous alleles were sequenced to verify allele lengths.

Statistical methods

Data from the questionnaires were entered twice using EpiInfo 6.04b software (US Centers for Disease Control and Prevention, Atlanta, Ga), validated and together with data from *TNFA* SNP typing, Sequenom MassArray typing and fragment length analysis imported into SPSS version 11.5 (SPSS Inc, Chicago, Ill) for statistical analysis. The Hardy-Weinberg equilibrium of each polymorphism was checked in the total population and in each group of respondents using the program HWE [25], *IFNGR1* CA repeat alleles with < 5% frequency were grouped for this computation. For the comparisons of the proportion, either the Pearson's Chi-Square test or Fisher's exact test was used. Association analyses of the CA repeat polymorphism were carried out using the program CONTING v2.62 [25].

Results

Cases of typhoid fever and paratyphoid fever

In all, 90 bacteremic typhoid fever patients and 26 bacteremic paratyphoid fever patients were enrolled in this study. The demographic characteristics of the cases are given in **Table 1**; only about 13 percent of the typhoid fever and paratyphoid fever cases were hospitalized, all other patients were treated in an outpatient setting.

Fever controls and randomly selected community controls

We included 337 fever controls. Of 378 randomly selected community controls, 56 (15%) refused to give blood. Sixty-two (19%) of the 322 community controls had a self reported history of typhoid fever; in none of these cases, however, was that history at the time

confirmed by a positive blood culture.
The gender distribution of the typhoid and paratyphoid cases was about even: 57:59 for the female and male, respectively; in the randomly selected community controls it amounted to 179:143. The difference of gender distribution between cases and community controls is not significant (p=0.37). The median age of cases, 20 years [Inter Quartile Range: 12-26.8], was similar to that of fever controls, both being significantly lower than that of the random community controls, i.e., 31.5 years [IQR: 18-49]. Of note, the age of typhoid fever cases did not differ significantly from that of paratyphoid fever cases (p=0.10). The population of Jatinegara is a mixture of Indonesians from different islands of the archipelago and in individual cases it is not possible to designate a subject to one group, or exclude admixture with certainty. However, based on the sublocation in the area and the subjects' names the ethnic makeup of our three study populations did not differ; no stratification with respect to possible admixture was made. In the non-enteric fever control group, patients could be infected with various bacterial and viral pathogens, each having distinct disease mechanisms. Therefore, the underlying genetic susceptibilities for this group could also be diverse. Although the inclusion of a fever control group would not provide a consistent reference group, we decided to present the findings in this group as well to further illustrate allelic frequencies in the population. Some additional characteristics of the respondent groups in the study can be found in **Table 1**.

Hardy-Weinberg equilibrium calculation

The single nucleotide polymorphisms (SNPs) analyzed in this study are summarized in **Table 2**. We started with calculating the Hardy-Weinberg equilibrium for all polymorphisms. The prevalence of the *TNFA–238* and *TNFA–308* genotypes in the total population group, case group and fever control group were in equilibrium. However, they are not

Table 1. Demographic data of typhoid fever and paratyphoid fever cases, community controls, and fever controls

	Cases (n=116)	Community controls (n=322)	Fever controls (n=337)
Age, median [IQR]	20 [12-26.8]	31.5 [18-49]	21 [14-30.5]
Gender, F/M	57/59	179/143	156/181
Low Family income (%) [a]	58 (51)	148 (46)	197 (59)
Household size, median (range) [b]	6 (1-200)	6 (1-50)	6 (1-20)
Hospitalized (%) [c]	15 (12.9)	N.A.	39 (11.7)
Days of inactivity (range)	10.9 (0-36)	N.A.	7.1 (0-50)

a Defined as below the median monthly income of the community controls (900.000 Rupiah [US$ 97])
b Includes 2 outliers: an orphanage with 200 individuals and a dormitory with 50 individuals in the typhoid fever cases and community controls, respectively.
c Respondents who were initially admitted to the hospital when the blood culture was taken or who were admitted to the hospital in later stage during the disease episode.

in equilibrium for the community control group (p<0.02 and p<0.01 for *TNA–238* and *TNFA–308*, respectively). This is due to the presence of 1 and 3 individuals with the AA genotype in the respective polymorphisms where the A allele proportion is very low. As the expected numbers of AA homozygotes are 0.1 and 0.8, we believe this is not a major deviation. The SNP *IFNG +874* was in equilibrium for the total population group, case group and community control group but not for the fever control group (p<0.02).

The two SNPs in *IL1R1* were nearly absent in our population (2 C alleles in 710 genotyped individuals and 2 G alleles in 728 genotyped individuals for *rs3917320* and *rs3917287*, respectively) and were therefore not studied further. All other polymorphisms studied were in Hardy-Weinberg equilibrium in the total population as well as in the separate groups of cases, fever controls and community controls.

Single Nucleotide Polymorphisms TNFA–238 and TNFA–308

The allele and genotype distributions for *TNFA–238* and *–308* in cases, randomly selected community controls and fever controls are given in **Table 3**. When comparing the allele distribution of *TNFA–238* between typhoid and paratyphoid fever patients and community controls, there was no significant difference (p=0.83). Similar findings were made when the typhoid fever cases only were compared with the community controls (p=0.75). Exclusion of the community controls with an unconfirmed history of typhoid fever did not change significantly the distribution of *TNFA–238* alleles in this group (p=0.55), nor did it affect the

Table 2 Distribution of alleles according to SNP database

Common SNP name	Official SNP designation	Frequency (%) of major allele in various populations [a] (Asian population)	Frequency (%) of major allele in Indonesian cohort
TNFA –238	rs361525	G 92-96 (n.a.)	G 98
TNFA –308	rs1800629	G 80-93 (n.a.)	G 95
IFNG +874	rs2430561	T n.a. [b]	T 68
IL1A –889	rs1800587	C 61-88 (88 in Chinese) G 92	
IL1B +3953	rs1143634	C 71-98 (98 in Chinese) C 97	
IL1B –511	rs16944	G 53-82 (53 in Japanese) G 55	
TNFRSF1A +36	rs767455	A 50-94 (94 in Asians [c]) A 89	
IL1R1 A124G	rs2228139	C 89-100 (100 in Asians [d]) C 100	
IL1R1 R456R	rs3917320	A 91-100 (100 in Chinese) A 100	
CASP1 codon 235	rs580253	C 73-100 (100 in Asians [d]) C 97	
CRP +1444	rs1130864	C 70-93 (n.a.)	C 90

a as obtained from the SNP database at www.ncbi.nlm.nih.gov
b present at 0.32-0.50 in various populations according to literature
c population not specified
d both Chinese and Japanese
n.a. not available

outcome of the comparisons with typhoid and paratyphoid fever patients taken together, or typhoid fever patients only (i.e., p= 0.51 and p=0.34, respectively).

Furthermore, the distribution of the TNFA–238 alleles was not significantly different in the fever control group as compared to enteric fever cases or typhoid fever patients only (p=0.54 and p= 0.37, respectively). Similar results were obtained upon comparison of the TNFA–308 genotype between the total number of enteric fever patients or typhoid fever case group only, and the community controls group (p=0.16 and p=0.11, respectively). Again, essentially identical results were obtained after exclusion of community controls with an unconfirmed history of typhoid fever (p= 0.29 and p=0.20 for typhoid and paratyphoid fever cases taken together, and typhoid fever only, respectively). Furthermore, the distribution of the TNFA–308 genotypes in the typhoid and paratyphoid cases group and fever controls group was not statistically significant (p=0.26 and p=0.13 for enteric fever case group and typhoid fever only, respectively).

We calculated odds ratios (OR) upon comparison of the alleles or genotypes of *TNFA–238* and *–308* in typhoid fever and paratyphoid fever cases with community controls and fever controls. For the *TNFA–238* polymorphism, we could not find an association of a particular allele or genotype and susceptibility or resistance against community acquired typhoid and paratyphoid fever or typhoid fever. For the *TNFA–308* polymorphism, in contrast to a previous study that associated allele A with the disease [5], the allele G appeared to be significantly associated with occurrence of typhoid and paratyphoid fever or typhoid fever specifically (OR = 4.42 [95% CI: 1.11-38.41] and OR = 2.87 [95% CI: 1.00-11.28], respectively). However, this association does no longer reach a level of significance when the comparison is made against the randomly selected community controls that did not have an -unconfirmed- history of typhoid or paratyphoid fever.

SNPs in IFNG, IL1A, IL1B, TNFRSF1A, CASP1 and CRP

The majority of the SNPs in this study were analyzed using a Sequenom MassArray system, which fully automatically determines the genotypes. There were no significant differences when we compared the genotypes or alleles of the SNPs in *IFNG*, *IL1A*, *IL1B*, *TNFRSF1A*, *CASP1* or *CRP* between typhoid and paratyphoid fever cases and community controls. Similar findings were made when the typhoid fever cases only were compared with the community controls. Exclusion of the community controls with an unconfirmed history of typhoid fever did not change significantly the distribution of the alleles in these groups. One of the SNPs, *IFNG +874* was not in Hardy-Weinberg equilibrium within the fever control group which may indicate the presence of an association between the cause of the fever in this group and certain genotypes. This was not further studied since we did not determine the causative

Table 3 Allele and genotype distributions of SNPs in pro-inflammatory genes

		Cases	Community controls	Fever controls
SNP	Alleles/ genotypes			
TNFA –238	A	0.02	0.02	0.02
	G	0.98	0.98	0.98
	AA	0 (0 %)	1 (0.3 %)	0 (0 %)
	AG	4 (3.6 %)	11 (3.4 %)	11 (3.3 %)
	GG	107 (96.4 %)	308 (96.3 %)	320 (96.7 %)
TNFA –308	A	0.02	0.05	0.03
	G	0.98	0.95	0.97
	AA	0 (0 %)	3 (0.9 %)	0 (0 %)
	AG	4 (3.6 %)	26 (8.1 %)	23 (6.9 %)
	GG	107 (96.4 %)	291 (90.9 %)	308 (93.1 %)
IFNG +874	A	0.31	0.32	0.38
	T	0.69	0.68	0.63
	AA	11 (10.5 %)	26 (8.6 %)	54 (17.3 %)
	AT	44 (41.9 %)	144 (47.4 %)	126 (40.4 %)
	TT	50 (47.6 %)	134 (44.1 %)	132 (42.3 %)
IL1A -889	A	0.11	0.08	0.09
	G	0.89	0.92	0.91
	AA	0 (0 %)	1 (0.3 %)	3 (0.9 %)
	AG	23 (21.9 %)	44 (14.4 %)	52 (16.2 %)
	GG	82 (78.1 %)	260 (85.2 %)	266 (82.9%)
IL1B +3953	A	0.04	0.03	0.03
	G	0.96	0.97	0.97
	AA	0 (0 %)	0 (0 %)	0 (0 %)
	AG	9 (8.6 %)	17 (5.6 %)	18 (5.6 %)
	GG	96 (91.4 %)	288 (94.4 %)	303 (94.4 %)
IL1B -511	A	0.40	0.45	0.45
	G	0.60	0.55	0.55
	AA	13 (12.5 %)	62 (20.3 %)	56 (17.7 %)
	AG	57 (54.8 %)	147 (48.4 %)	172 (54.4 %)
	GG	34 (32.7 %)	95 (31.3 %)	88 (27.9 %)
TNFRSF1A +36	C	0.09	0.11	0.10
	T	0.91	0.89	0.90
	CC	2 (1.9 %)	4 (1.4 %)	0 (0 %)
	CT	14 (13.3 %)	54 (18.3 %)	61 (19.2 %)
	TT	89 (84.8 %)	237 (80.3 %)	256 (80.8 %)
CASP1 CODON 235	A	0.02	0.03	0.03
	G	0.98	0.97	0.97
	AA	0 (0 %)	0 (0 %)	1 (0.3 %)
	AG	4 (3.8 %)	19 (6.2 %)	19 (5.9 %)
	GG	100 (96.2 %)	287 (93.8 %)	302 (93.8 %)
CRP +1444	T	0.09	0.10	0.11
	C	0.91	0.90	0.89
	TT	0 (0 %)	5 (1.7 %)	6 (1.9 %)
	CT	20 (18.7 %)	49 (16.7 %)	57 (17.8 %)
	CC	87 (81.3 %)	239 (81.6 %)	257 (80.3 %)

bacterial strains in this group other than exclusion of *S. (para)typhi*.
The distribution of alleles and genotypes of these SNPs are shown in **Table 3**.

Variable length polymorphisms in IFNGR1 and IL12B

There were no significant differences when we compared the genotypes or alleles of the CA repeats in *IFNGR1* and the ins/del polymorphism in *IL12B* between enteric fever cases and community controls. Also no significant differences were found between the fever controls and the enteric fever cases. The distribution of the alleles is shown in **Table 4**.

Discussion

The main finding of the present study is that polymorphisms in a series of pro-inflammatory genes were not associated with typhoid fever and thus appear not to contribute to susceptibility to acquire typhoid fever unlike the previously described polymorphisms in *CFTR* and *PARK2/PACRG* in the same typhoid fever cohort [26,27]. In view of earlier findings the *TNFA–308* polymorphism, however, might be related to severity of established disease rather than to susceptibility per se.

To analyze association of polymorphisms with susceptibility to typhoid fever and paratyphoid fever, we compared the prevalence of the polymorphisms in typhoid and paratyphoid cases with those of fever controls and community controls. Some of the potential pitfalls that may affect complete enrollment of patients, most of whom were recruited in outpatient facilities in the area, and the classification of cases and fever controls have been described in detail elsewhere [2]. In short, provisions were taken to minimize misclassification of patients, including stool cultures 3 to 4 weeks after the blood culture, when *S. typhi* and *S. paratyphi A* may still be excreted in the feces of patients [28]. For every patient with typhoid fever or paratyphoid fever, we included 4 controls of two groups recruited from the same study area: fever controls who like typhoid fever patients presented with 3 days of fever but of whom blood cultures either showed no growth or growth of bacteria other than Salmonellae, or randomly selected community controls. Although the fever controls likely suffered from a divergent spectrum of diseases other than enteric fever, and as such do not constitute a consistent reference group as the random community controls do, we decided to include the findings in this group to further illustrate the allelic frequencies found in the Indonesian population. Nineteen percent of the randomly selected community controls reported a possible episode of typhoid fever in the past. Likely, this percentage is an overestimation of the real number of cases since most fever patients are empirically treated in outpatient clinics without confirmatory diagnosis, but importantly,

the distribution of polymorphisms in the community control group was not significantly different when these community controls were left out of the analysis. The age of the typhoid cases and the random community controls did differ, whereas the incidence of typhoid is higher in the age group below twenty. In this respect, however, the distribution of polymorphisms in the community control group did not differ for different age cohorts, e.g., those under or above their 20s. Although the sample size may have influenced the outcome to some extent we have more than 80% power to detect significant differences with an OR of 3 in e.g. *TNFA-308* allele distribution, this should have provided sufficient power to replicate the findings in Vietnam where an OR of >5 was found [5].

The role of pro-inflammatory proteins like TNF-α, IFN-γ, IL-1α, IL-1β, IL-12, TNFR1, IFN-γR1, IL-1R1, CASP1 and CRP in induction of expression of a variety of genes and the synthesis of several proteins that induce acute and chronic inflammatory changes are well established [19;19;29]. Although polymorphisms in some of these genes were found to

Table 4 Allele distribution of IFNGR1 CA repeat and IL12B ins/del polymorphisms

		Cases	Community controls	Fever controls
POLYMORPHISM	ALLELES OR GENOTYPES			
IFNGR1 CA	CA_{11}	1 (0.4 %)	0 (0 %)	0 (0 %)
	CA_{12}	82 (35.7 %)	183 (28.5 %)	211 (31.4 %)
	CA_{13}	0 (0 %)	1 (0.2 %)	0 (0 %)
	CA_{16}	5 (2.2 %)	18 (2.8 %)	21 (3.1 %)
	CA_{17}	4 (1.7 %)	16 (2.5 %)	11 (1.6 %)
	CA_{18}	32 (13.9 %)	109 (17.0 %)	96 (14.3 %)
	CA_{19}	27 (11.7 %)	66 (10.3 %)	81 (12.1 %)
	CA_{20}	7 (3.0 %)	32 (5.0 %)	27 (4.0 %)
	CA_{21}	1 (0.4 %)	11 (1.7 %)	16 (2.4 %)
	CA_{22}	22 (9.6 %)	65 (10.1 %)	57 (8.5 %)
	CA_{23}	31 (13.5 %)	78 (12.1 %)	82 (12.2 %)
	CA_{24}	16 (7.0 %)	61 (9.5 %)	63 (9.4 %)
	CA_{25}	2 (0.9 %)	2 (0.3 %)	7 (1.0 %)
	total	**230**	**642**	**672**
IL12B ins/del [a]	short allele	127 (56.2 %)	336 (53.2 %)	382 (57.4 %)
	long allele	99 (43.8 %)	296 (46.8 %)	284 (42.6 %)
	short/short	38 (33.6 %)	81 (25.6 %)	108 (32.4 %)
	short/long	51 (45.1 %)	174 (55.1 %)	166 (49.8 %)
	long/long	24 (21.2 %)	61 (19.3 %)	59 (17.7 %)

a The short allele contains GC that in the long allele is replaced by CTCTAA, resulting in a 4 nt difference in length.

be associated with susceptibility to infectious disease like tuberculosis [7,9], we failed to associate these polymorphisms with manifest infection by the intracellular pathogens *Salmonella typhi* and *Salmonella paratyphi* A.
Unlike in Vietnam, in Indonesia the prevalence of the –238 and –308 polymorphisms in the promoter region of the gene encoding TNF-α in patients with typhoid and paratyphoid fever does not differ significantly from those in fever controls or community controls. This discrepancy might be explained by the way the patients were selected for the respective studies, i.e., use of typhoid fever patients admitted to hospital in Vietnam [5] as compared with consecutive (para-) typhoid fever patients enrolled in the community-based surveillance study in Jakarta. In our study, only a minority of the patients (~13 %) was hospitalized. Patients admitted to hospital usually have more severe disease than patients treated in an outpatient setting. Together, the findings suggest that *TNFA* promoter polymorphisms, the *TNFA–308* in particular, may have a role not in susceptibility to acquire (para-)typhoid fever but in determining the course and severity of the established disease requiring hospitalization of patients. Such an explanation is consistent with earlier findings that the *TNFA–308*A* allele was associated with severity and mortality of meningococcal infection [30], and bacterial sepsis [31], cerebral malaria [32], mucocutaneous leishmaniasis [33], as well as some viral diseases [34] and trauma [30-33,35,36]. Although the presence of *TNFA–308*A*, may result in somewhat higher constitutive and inducible levels of gene transcription than *TNFA–308*G* [37,38] in clinical practice it cannot be determined readily whether greater TNF-α production causes a more severe inflammatory response or, conversely, whether more severe inflammation elicits greater TNF-α synthesis [39].

Due to the lack of a suitable animal model for *S. typhi* infection the pathogenesis of typhoid fever has not been studied extensively. Gene expression profiling of human model intestinal cells infected with *Salmonella typhi* has shown that *S. typhi*, in contrast to a well-defined pro-inflammatory pathogen like *S. typhimurium*, does not elicit a pro-inflammatory response [40]. In human colonic tissue explants infected with *S. typhi* it was found that the Vi antigen expressed by *S. typhi* is able to reduce TLR5 and TLR4 mediated responses which may account for the lack of inflammatory infiltrates in the human intestinal mucosa [41]. Both these *in vitro* experiments and our genetic data suggest that the role of pro-inflammatory cytokines may be limited in determining the outcome of *S. typhi* infection. We recently found an association of susceptibility to typhoid fever (OR 2.6) with polymorphisms in *CFTR*, which encodes for a protein expressed on the intestinal epithelium that is utilized by *S. typhi* to enter the epithelial cells before entering the bloodstream [26]. A crucial step in developing typhoid fever is the ability of the bacteria to penetrate the gut epithelium and this may mean that the genes that will be found to influence the susceptibility to typhoid fever will be encoding proteins that are expressed in the gut epithelium and are involved in

S. typhi entry or passage. Once the salmonellae have passed the epithelial layer and entered the bloodstream the pro-inflammatory genes may merely be able to influence disease severity and speed of recovery.
Two SNPs (in *IL1R1*) of which the minor alleles were reported with a low frequency in the SNP database were virtually absent in the Indonesian population. Most of the other SNPs in the pro-inflammatory genes we studied proved to have a low frequency of the minor allele: *TNFA-238*, *TNFA-308*, *IL1A -889*, *IL1B +3953*, *TNFRSF1A +36* and *CASP1 codon 235* (**Table 2**). No Indonesian panel has thus far been used for establishing allele frequencies for the SNP database. Before embarking on this study we therefore did not know whether allele frequencies would be similar to other Asian populations or would diverge greatly. To have the necessary power to detect an association with polymorphisms of which the minor allele frequency is < 0.05 (as was the case for *TNFA –238*, *IL1B+3953* and *CASP1 c235* in this population) a study with a larger sample size is required. Ideally, one would want to analyze SNPs of which the allele frequencies have been validated in the population that one is about to study, these may however not be the polymorphisms one would prefer to analyze based on functional data (expression) and association studies from the literature.

In conclusion, we did not find an association of susceptibility to typhoid and paratyphoid fever and *TNFA* promoter polymorphisms as was previously described for the *TNFA–308*A* allele and suggest that this polymorphism might be related to severity of established disease rather than to susceptibility per se. We also did not find an association between a series of other pro-inflammatory genes and conclude that polymorphisms in pro-inflammatory genes appear not to contribute to susceptibility to typhoid fever.

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