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Variation in the *TLR10/TLR1/TLR6* Locus is the Major Genetic Determinant of Inter-Individual Difference in TLR1/2-Mediated Responses

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Abstract

Toll-like receptor (TLR)-mediated innate immune responses are important in early host defense. Using a candidate gene approach, we previously identified genetic variation within *TLR1* that is associated with hyper-responsiveness to a TLR1/2 agonist *in vitro* and with death and organ dysfunction in patients with sepsis. Here we report a genome-wide association study designed to identify genetic loci controlling whole blood cytokine responses to the TLR1/2 lipopeptide agonist, Pam_3CSK_4 *ex vivo*. We identified a very strong association ($p<1\times10^{-27}$) between genetic variation within the *TLR10/1/6* locus on chromosome 4, and Pam_3CSK_4-induced cytokine responses. This was the predominant association explaining over 35% of the population variance for this phenotype. Notably, strong associations were observed within *TLR10* suggesting genetic variation in *TLR10* may influence bacterial lipoprotein-induced responses. These findings establish the *TLR10/1/6* locus as the dominant common genetic factor controlling inter-individual variability in Pam_3CSK_4-induced whole blood responses in the healthy population.

Keywords

TLR; polymorphism; genomics; innate immunity

Introduction

The innate immune system provides early recognition of microbial pathogens important to host defense. Toll like receptors (TLRs) play a key role in host defense, providing a mechanism to respond to highly conserved pathogen-associated molecular patterns

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Conflicts of Interest

The authors declare no conflict of interest.

(PAMPs).[1] In humans, there are ten unique TLR genes coding for receptors that initiate responses to PAMP ligands a robust inflammatory response. TLR2 heterodimerizes with TLR6, TLR1, and possibly TLR10, and these combinations facilitate the recognition of multiple distinct bacterial patterns diversifying innate immune sensing.[2–4] The importance of TLR2 in host defense has been well-established in mice where its deficiency has been associated with increased susceptibility to mycobacterial infection, pneumococcal meningitis, and sepsis due to *Staphylococcus aureus* and *Listeria monocytogenes.*[5–8] TLR1/2 and TLR2/6 heterodimers can discriminate the acylation of bacterial lipopeptides recognizing triacyl- and diacyl-lipopeptides respectively.[2,9–11] The synthetic triacyl lipopeptide N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-$(Lys)_4$ (Pam_3CSK_4) and diacyl lipopeptide Fibroblast Stimulating Ligand-1 (FSL-1) derived from *Mycoplasma salivarium* have been shown to stimulate via TLR1/2 and TLR2/6 heterodimers.[2,12] Additionally, TLR2/6 heterodimers recognize peptidoglycan (PGN) and a yeast cell wall particle, Zymosan.[13,14]

A role for TLR1/2 and TLR2/6 in human disease has been suggested by candidate gene studies. We and others have demonstrated that there exists high inter-individual variability in terms of human leukocyte inflammatory responses to PAMPs[15,16] and that a portion of this variability is attributable to common genetic variants. Genetic variation in *TLR2* has been shown to confer reduced responses to peptidoglycan and heat-killed *S. aureus in vitro*.[17] More recently, we have demonstrated that variants in *TLR1* are highly associated with Pam_3CSK_4-induced whole blood cytokine production. We reported that common genetic variants in *TLR1* conferred marked hyper-responsiveness to Pam_3CSK_4 and these same variants were associated with increased risk of organ dysfunction and death in septic shock.[15,18] Other studies have demonstrated associations between genetic variation in *TLR1* with susceptibility to leprosy and tuberculosis.[19,20] These data support a role for TLR1/2-mediated responses in human disease. However, to date, our understanding of the role for genetic variation in TLR-mediated responses has been based on targeted candidate gene studies. Thus, in order to more comprehensively assess the genetic factors controlling TLR2-mediated responses in the healthy human population we undertook a genome wide association study to identify loci modifying Pam_3CSK_4-induced cytokine production in whole blood *ex vivo*.

Results

We employed samples from 360 healthy Caucasian subjects who had an average age of 35±14 years and were 39% male. Given that many innate immunity genes demonstrate population differences in allele frequencies including the genes coding for TLRs,[21] we performed principal components analysis (PCA) to address the possibility that there might exist population admixture within our genotyped subjects. PCA revealed that subjects who self-reported as Caucasian cluster with Caucasians from Utah (CEU) and the Toscani in Italia (TSI) populations from the HapMap3 collection[22] (Supplemental Figure 1). However, we did identify associations between eigenvalues from the first three principal components and TLR agonist-induced cytokine production and so these eigenvalues were used as covariates in the multiple linear regression models for the GWAS.

We used a genome wide association test adjusted for age, gender and the first 3 principal components, and identified 19 SNPs within the *TLR10/1/6* locus on chromosome 4 that were associated with Pam_3CSK_4 induced IL-6 (Figure 1A), IL-1β, and TNF-α production in whole blood (Supplemental Figure 2) at a genome-wide level of significance ($p = 1 \times 10^{-8}$ – $p = 1 \times 10^{-27}$) (Table 1). No other loci achieved associations at a genome-wide level of significance including SNPs found in genes involved in TLR1/2 signaling such as *TIRAP*, *IRAK4*, and *IRAK1* that we had anticipated *a priori* would be associated with the cytokine induced phenotypes (Table 2). Notably, all cytokine values obtained from the whole blood assay were normalized to a monocyte count obtained from the donor at the time of phlebotomy. In this way we mitigated the chances of identifying variation that merely affected the number of circulating monocytes.

We next sought to identify loci associated with responses to TLR2/6 ligands FSL-1, PGN, and Zymosan in 167 subjects for whom we had measured whole blood responses to these ligands. We did not identify any associations reaching genome-wide significance (Figure 1B) and, notably, no SNPs within the *TLR10/1/6* locus or *TLR2* were even nominally associated ($p>0.05$) with responses to these ligands. Nonetheless, there were several moderately strong associations detected at other genomic loci with these cytokine responses ranging from $p=1.55\times10^{-6}$ (Zymosan-induced IL-6), $p=3.30\times10^{-6}$ (FSL-induced IL-6) to $p=4.37\times10^{-6}$ (PGN-induced IL-6). Since these analyses included fewer subjects than the GWAS of Pam_3CSK_4-induced responses we re-ran the GWAS of Pam_3CSK_4–induced IL-6 using only these 167 subjects. This analysis still identified multiple SNPs that were associated at a genome-wide level of significance ($p<4.7\times10^{-12}$) demonstrating that while statistical power for this sub-study may have been limiting, the associations with Pam_3CSK_4–induced responses are orders of magnitude stronger than any associations with TLR2/6 agonist-induced responses.

In order to identify SNPs within the *TLR10/1/6* locus not directly genotyped by our platform that may be driving the observed associations with Pam_3CSK_4-induced cytokine production, we used imputation to infer missing genotypes on chromosome 4 using 1000 genomes NCBI Build 37[23] as a reference population. These imputed SNPs were tested for association with the Pam_3CSK_4-induced cytokine phenotypes. We observed a 222kb region across the *TLR10/1/6* locus that was associated with Pam_3CSK_4-induced IL-6 at a genome wide level of significance (Figure 2). The SNP most highly associated with hypermorphic responses was rs67719080 ($p=1\times10^{-27}$), an intergenic SNP between *TLR10* and *TLR1*. Of the SNPs that fell within genes, SNPs within *TLR10* were most highly associated with hypermorphic cytokine responses (Figure 2). The most highly associated *TLR10* coding SNP was rs4129009 ($TLR10_{2323A/G}$), a non-synonymous polymorphism that causes an amino acid change in the highly conserved Toll/Interleukin-1 receptor (TIR) domain. Individuals homozygous for the rare allele had increased IL-6 production consistent with a hypermorphic response (Figure 3). In addition to the TIR domain SNP, we also identified a missense SNP in *TLR10*, rs11096955 (I369L), near leucine-rich repeat 9 (LRR9: aa 349–368) of *TLR10* that was strongly associated with hypermorphic responses to Pam_3CSK_4 ($p=5.36\times10^{-16}$).

Coding SNPs within *TLR1* were also highly associated with the Pam_3CSK_4-induced cytokine phenotype including rs4833095 ($TLR1_{742A/G}$) and rs5743618 ($TLR1_{1805G/T}$) but were not in high LD with the TLR10 coding SNP rs4129009 (Table 3) suggesting a distinct association. Notably, rs5743551 a SNP found 5′ to *TLR1* that we have previously shown to be highly associated with death and organ dysfunction in sepsis was also highly associated ($p=2.8\times10^{-24}$). Finally, we also found a strong association with a non-synonymous variant in *TLR6* (rs5743818, $TLR6_{1932T/G}$) and Pam_3CSK_4-induced responses ($p=1.28\times10^{-9}$). This SNP was not found to be in high linkage disequilibrium with the other most-highly associated coding SNPs in *TLR1* ($R^2=0.11$) and *TLR10* ($R^2=0.08$) (Table 3).

Discussion

In this genome-wide association study, we found that the *TLR10/1/6* region on chromosome 4 is the dominant common genetic locus controlling inter-individual variation in responses to Pam_3CSK_4 in whole blood from healthy subjects *ex vivo*.

While the genes coding for TLRs are distributed throughout the genome, *TLR10*, *TLR1*, and *TLR6* cluster at a locus on chromosome 4p14. Evidence suggests that this tandem arrangement arose from a gene duplication event.[24] Notably, all three of these genes have significant allelic heterogeneity with an abundance of rare variants that may indicate an influence of purifying selection.[24] In addition, there exist significant geographic differences in genetic variation between European populations within the *TLR10/1/6* locus.[21] However, our principal components analysis shows that our subjects clustered with Caucasian populations in HapMap3 and our adjustment with principal components in the linear regression suggests that the association testing is not confounded by cryptic population substructure.

Among the SNPs within *TLR1* showing the strongest associations in our study were several that have been previously associated with susceptibility to leprosy (rs5743618)[25], risk for prostate cancer and placental malaria (rs4833095).[26,27] These findings are consistent with the assertion that functional responses mediated by TLR1/2 heterodimers might drive important biologic responses and alter risk for disease. We were more surprised to find strong associations with coding SNPs within *TLR10* as there is no known ligand specific for TLR10 and it is not known that TLR10 ligation actually generates an intracellular response.[4,28] These findings suggest that SNPs within *TLR10* may contribute to associations between disease susceptibility and the *TLR10/1/6* locus.

The most highly associated non-synonymous SNP in *TLR10*, rs4129009 causes an amino acid change in the TIR domain of the intracellular portion of the protein. The TIR domain is critical for intracellular signaling in other TLR family members.[29,30] A recent study has shown that a chimeric receptor containing the extracellular domain of *TLR10* and the intracellular domain of *TLR1* (including the TIR domain) induced a cellular response to Pam_3CSK_4 comparable to wild-type *TLR1*.[4] This study suggests that the extracellular portion of TLR10 recognizes Pam_3CSK_4 but that the intracellular portion of TLR10 does not translate this recognition event to an intracellular signal. Our study shows that individuals homozygous for the rare allele of rs4129009 in *TLR10* have increased cytokine

responses suggesting that this genetic alteration of the TIR domain may result in a functionally active TLR10 molecule. Of note, this SNP has previously been reported to be associated with decreased risk of atopic asthma.[31] In addition to this SNP in the TIR domain, we identified another highly associated missense SNP in *TLR10*, rs11096955 (I369L), near LRR9, that could alter ligand binding. In order to best identify whether the *TLR10* signal is an independent association, future research should be aimed at other racial groups where haplotype blocks in these region are smaller. Future work will need to more finely delineate whether SNPs in *TLR10* or *TLR1* (or both) are causally responsible for the associations observed. However, due to moderate LD, conditional regression analysis adjusting for the top SNPs in this analysis was underpowered to detect independent associations.

The importance of genetic variation in TLR genes and downstream TLR signaling genes is highlighted by candidate gene studies that have demonstrated associations between variants in these genes and diseases for which host defense and inflammation is pathologic. With respect to genes encoding the TLR1/2 heterodimer, functional polymorphisms within the *TLR10/1/6* locus and *TLR2* have been associated with altered susceptibility to the mycobacterial infections of leprosy and tuberculosis.[19,20,32] A *TLR1* polymorphism (rs5743618, Ser602Ile) that mediates higher levels of signaling and cell surface expression[15,19] is associated with protection from recurrent urinary tract infection and pyelonephritis.[33] In sepsis, where severe infection leads to overwhelming inflammation and end-organ dysfunction, a *TLR 1* polymorphism (rs5743551) associated with marked hyper-responsiveness has been associated with risk of death and organ dysfunction and sepsis induced acute lung injury.[15,18] Outside of infectious diseases, polymorphisms within the *TLR10/1/6* locus have been variably associated with prostate cancer, non-Hodgkin lymphoma, Crohn's disease, asthma, and chronic sarcoidosis.[26,31,34–40] Our findings that the *TLR10/1/6* locus explains a large portion of population variance in TLR1/2-mediated responses *in vitro* provides additional support for the importance of this locus in human disease.

Several previous reports have demonstrated associations between disease risk and genetic variation in TLRs and genes of the TLR intracellular signaling pathway including *TLR2*, *TIRAP*, *IRAK4*, and *IRAK1*.[41–43] In spite of these previous findings, we detected only a nominally significant association with variants in some TLR-related genes (Table 2). It should be noted that this study was designed to have adequate statistical power to detect associations with common genetic factors (MAF >5%). This study is inadequately powered for detection of associations with rare genetic variants (MAF<1%) and, therefore, we cannot exclude the possibility that rare variants within these or other genes may also play a role in modulating these effects. Nonetheless, our findings suggest that common genetic variation in TLR pathway genes outside of the *TLR10/1/6* locus play only a minor role in modifying TLR1/2 responses in the Caucasian population.

In summary, our study shows that genetic variation within the *TLR10/1/6* locus is the major common genetic factor explaining inter-individual variation in TLR1/2-mediated cytokine responses to Pam_3CSK_4 *in vitro*. We find that the mostly highly-associated SNPs fall within *TLR10* and that some of these SNPs are located in or near important functional domains

(TIR domain and LRR9) of TLR10 suggesting that this receptor might have functional relevance. Overall, this study supports ongoing efforts to understand the importance of this locus to human diseases involving innate immunity.

Materials and Methods

Study Subjects

We used DNA samples and innate immune response phenotypes collected from 360 healthy Caucasian volunteers recruited from the Seattle metropolitan area from whom written informed consent was obtained. This was approved by the University of Washington Human Subjects Committee. This population has been previously described by our group.[15]

Cytokine Assays

Innate immune responses were measured in whole blood *ex vivo* as previously described.[16] We exposed whole blood collected from each subject to Pam_3CSK_4 (360 subjects at 100ng/ml), FSL-1, PGN (167 subjects at 100ng/ml) and Zymosan (179 subjects at 100μg/ml) for six hours, supernatants were collected, and production of Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Granulocyte colony stimulating factor (G-CSF), Interleukin 1-receptor antagonist (IL-1ra), monocyte-chemotactic protein-1 (MCP-1) was measured by cytometric bead-based immunoassay (Luminex™). A complete blood count with differential cell counts was obtained at the time of blood sampling for the stimulation assays and cytokine concentrations were normalized to monocyte counts.

Genotyping and Imputation

Genomic DNA was genotyped using the Illumina™ Human 1M Beadchip array. In addition, we imputed genotypes on chromosome 4 not present on the array with the BEAGLE software package version 3.3[44] using EUR genotypes from 1000 Genomes[23] as a reference.

Quality Control

Quality control was performed as described by Anderson et al.[45] We assessed for discordance between reported sex and genotype-determined sex, excess autosomal heterozygosity, excess relatedness (identity by descent of > 0.1875), and population substructure using principal components analysis (PCA) and removed 14 subjects resulting in a total of 346 subjects. All subjects had a genotype call rate of over 97%. The 561,491 SNPs were filtered to remove all SNPs with a minor allele frequency (MAF) <0.05, Hardy-Weinberg equilibrium $p<0.001$, or a call rate ≤ 0.90 resulting in 493,197 SNPs that were used for association testing. Imputed SNPs for chromosome 4 were filtered for an allelic R^2 of 0.85.

Data analysis

We tested for associations between genome-wide genotypes and $\log_{10}$-transformed, monocyte normalized, cytokine values by multiple linear regression assuming additive effects. Subjects and SNPs passing QC filtering were tested for association with Pam_3CSK_4-

induced, monocyte-normalized, whole blood cytokine production adjusting for covariates including age, gender and eigenvalues from the first three principal components generated by PCA clustering subjects with samples from HapMap3 (Release 3, NCBI build 36).[22] Correcting for multiple tests, we considered a $p < 1 \times 10^{-8}$ to be indicative of genome-wide significance. We assigned p-values to TLR signaling genes anticipated *a priori* to be associated with the cytokine phenotype by choosing the p-value of the highest SNP within a 50kB range from the 5′ and 3′ end of the gene. All above analyses were performed and linkage disequilibrium calculated using the Golden Helix™ software package.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used is this paper

Pam_3CSK_4	N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-$(Lys)_4$
FSL-1	Fibroblast Stimulating Ligand-1
PGN	peptidoglycan
PCA	principal components analysis
TIR	Toll/Interleukin-1 receptor
LRR	leucine-rich repeat

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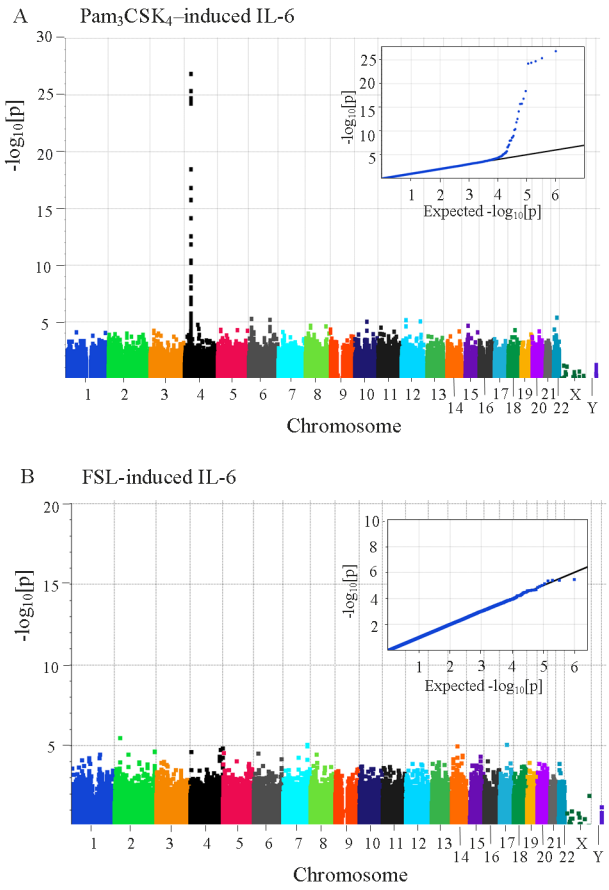

Figure 1.

The *TLR10/1/6* locus is highly associated with Pam_3CSK_4-induced IL-6. A) Manhattan plot showing the primary association statistics for the Pam_3CSK_4–induced IL-6 concentration across all chromosomes for the 483,197 genotyped SNPs. Embedded quantile-quantile plot of $-\log_{10}$(P value) vs. the expected $-\log_{10}$(P value) for SNP associations with Pam_3CSK_4-induced IL-6 phenotype. B) Similar association statistics for FSL-induced IL-6 concentration.

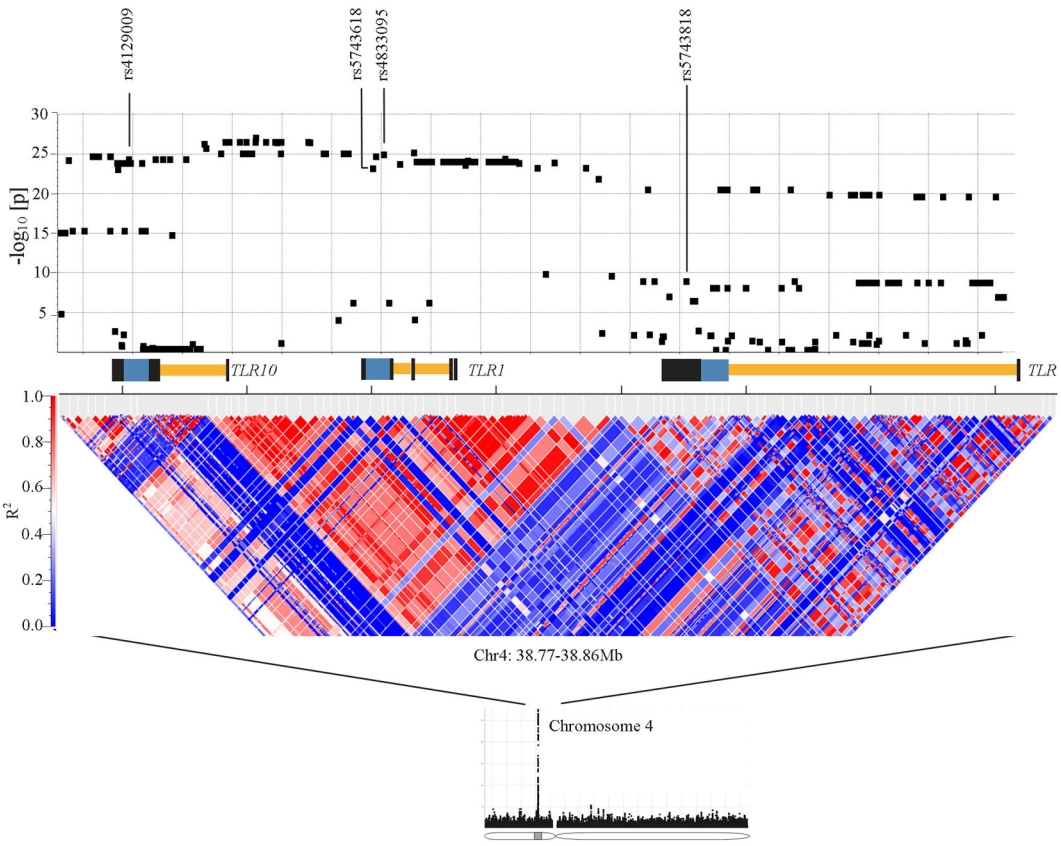

Figure 2.

Fine mapping of associations in *TLR10/1/6* locus with imputed genotypes. Association statistics for the imputed SNPs on chromosome 4 versus the $-\log_{10}$(P value) of the Pam_3CSK_4-induced IL-6 phenotype with associated LD plot. Area of focus is the *TLR 10/1/6* locus and highly associated SNPs are shown as black squares. The SNPs shown by rs number are the most highly associated coding SNPs in each gene. The lower plot shows all of chromosome 4 with the gray box representing cytoband 4p14.

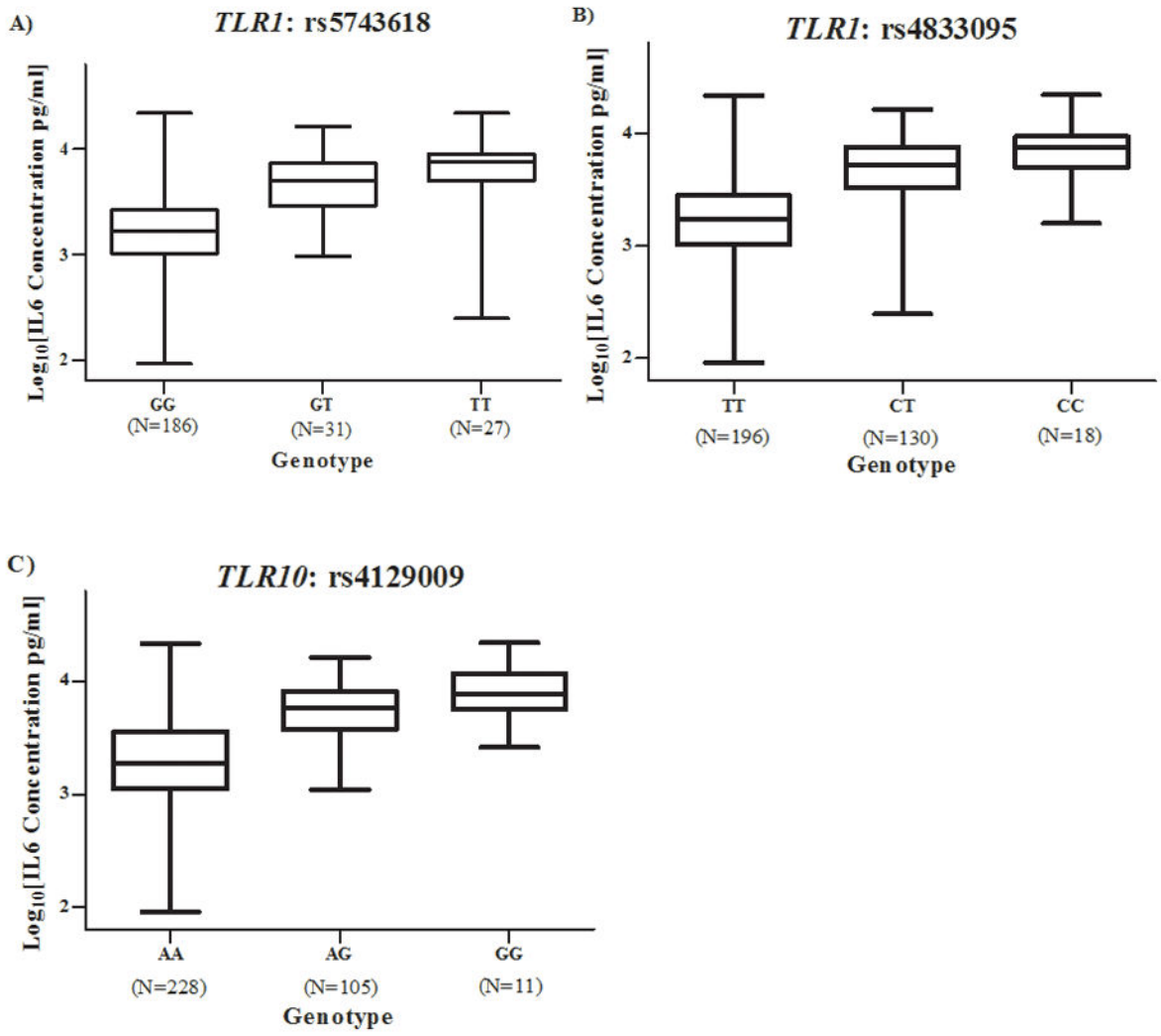

Figure 3.

Minor alleles in *TLR1* and *TLR10* are associated with hypermophic effects on Pam3CSK4-induced IL6. Coding SNPs for *TLR1* (A, B) and *TLR10* (C) most highly associated with Pam3CSK4-induced IL6 showing hypermorphic responses with the rare genotype.

Table 1

Top ranked associations with Pam_3CSK_4-induced IL-6[1]

SNP	P-value[2]	Gene	Left Gene	Right Gene	Allele	MAF	β[3]
rs4543123	1.35e-27	intergenic	*TLR10*	*TLR1*	C/T	0.23	+0.36
rs4833095	4.29e-26	*TLR1*	*TLR10*	*TLR6*	C/T	0.24	+0.35
rs11466640	1.85e-25	*TLR10*	*KLF3*	*TLR1*	C/T	0.18	+0.37
rs5743563	3.66e-25	*TLR1*	*TLR10*	*TLR6*	C/T	0.20	+0.36
rs11096956	5.57e-25	*TLR10*	*KLF3*	*TLR1*	G/T	0.22	+0.35
rs1873195	3.46e-19	*FAM114A1*	*TLR6*	*TMEM156*	A/G	0.22	+0.30
rs10008492	1.51e-17	intergenic	*KLF3*	*TLR10*	C/T	0.35	+0.26
rs4331786	1.72e-16	intergenic	*KLF3*	*TLR10*	A/G	0.31	+0.26
rs11096957	1.85e-16	*TLR10*	*KLF3*	*TLR1*	A/C	0.31	+0.26
rs10024216	7.25e-15	intergenic	*KLF3*	*TLR10*	A/G	0.30	+0.25
rs2890620	2.70e-13	intergenic	*KLF3*	*TLR10*	C/T	0.30	+0.24
rs6824105	1.45e-12	intergenic	*KLF3*	*TLR10*	A/G	0.29	+0.23
rs7660102	3.81e-11	intergenic	*KLF3*	*TLR10*	A/C	0.22	+0.22
rs4833103	5.87e-11	intergenic	*TLR1*	*TLR6*	A/C	0.49	+0.19
rs11944159	9.74e-10	*FAM114A1*	*TLR6*	*TMEM156*	C/T	0.27	−0.19
rs17429245	2.01e-09	intergenic	*TLR6*	*FAM114A1*	C/T	0.26	−0.19
rs6824001	2.53e-09	*FAM114A1*	*TLR6*	*TMEM156*	C/T	0.27	−0.18
rs13132956	8.93e-09	intergenic	*KLF3*	*TLR10*	C/T	0.20	+0.21
rs6833914	9.74e-09	intergenic	*TLR6*	*FAM114A1*	C/T	0.29	−0.18
rs4832792	1.05e-08	*FAM114A1*	*TLR6*	*TMEM156*	A/C	0.29	+0.18

[1] All SNPs meeting genome-wide significance (p<1e-8).

[2] Adjusted for gender and eigenvalues from first 3 principal components.

[3] Effect size and direction associated with copy number of minor allele (change in mean $\log_{10}$[IL6] with each copy of minor allele).

Table 2

Genes anticipated *a priori* to be associated with Pam_3CSK_4-induced IL-6 phenotype[1]

Gene[1]	Chromosome	Loci (Mb)[2]	SNP[3]	SNP Gene[4]	P-value
TLR2	4	154.84–154.85	rs2405432*	*RNF175*	0.17
NFKB1	4	103.54–103.76	rs2085548	intergenic	0.25
CD14	5	139.99–139.99	rs1583005	*IK*	0.02
MYD88	3	38.16–38.16	rs9825655	*DLEC1*	0.02
IRAK4	12	42.43–42.46	rs7972025	intergenic	0.04
LY96	8	75.07–75.10	rs10504553	intergenic	0.04
TIRAP	11	125.66–125.66	rs478309	*FOXRED1*	0.11

[1] TLR and TLR signaling genes anticipated to be associated with the agonist-induced cytokine concentration.

[2] For each gene, a window 50Kb from either end of the gene was included to select the most highly associated SNP.

[3] SNP most highly associated within the gene range. Asterisk signifies the SNP was imputed.

[4] Gene in which the SNP was located.

Table 3

Coding SNPs in *TLR10/1/6* locus most-highly associated with Pam_3CSK_4-induced responses[1]

SNP	P-Value[2]	Gene	Alleles	Feature	LD[3]
rs4833095	1.15e-25	*TLR1*	C/T	Missense: S248N	1.0
rs4129009	5.04e-25	*TLR10*	A/G	Missense	0.66
rs4274855	5.04e-25	*TLR10*	A/G	utr-5′	0.66
rs5743566	9.71e-25	*TLR1*	C/G	utr-5′	0.73
rs5743565	9.71e-25	*TLR1*	A/G	utr-5′	0.73
rs9715841	1.61e-24	*TLR10*	C/T	utr-3′	0.61
rs10776482	1.61e-24	*TLR10*	C/T	coding-synonymous	0.61
rs10776483	1.61e-24	*TLR10*	C/T	coding-synonymous	0.61
rs11096956	1.61e-24	*TLR10*	G/T	coding-synonymous	0.61
rs5743618	7.10e-24	*TLR1*	G/T	Missense: I602S	0.86
rs11466661	5.36e-16	*TLR10*	A/C	utr-3′	0.61
rs11096955	5.36e-16	*TLR10*	A/C	Missense: L369I	0.61
rs11096957	5.36e-16	*TLR10*	A/C	Missense: N241H	0.61
rs5743818	1.28e-09	*TLR6*	G/T	coding-synonymous	0.11

[1] Most highly associated coding SNPs to PAM3CSK4-induced IL-6.

[2] Adjusted for age, gender, and eigenvalues from first 3 principal components.

[3] Linkage disequilibrium (R^2) between each SNP and the highest TLR1 coding SNP rs4833095