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Replication of Associations between Cytokine and Cytokine Receptor Single Nucleotide Polymorphisms and Measles-Specific Adaptive Immunophenotypic Extremes

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Abstract

Our objective was to replicate previously reported associations between cytokine and cytokine receptor SNPs and humoral and CMI (cell-mediated immune) responses to measles vaccine. All subjects (n=758) received two doses of MMR (measles/mumps/rubella) vaccine. From these subjects, candidate cytokine and cytokine receptor SNPs were genotyped and analyzed in 29–30 subjects falling into one of four “extreme” humoral (Ab^{high/low}) and CMI (CMI^{high/low}) response quadrants. Associations between seven SNPs (out of 11 in the discovery study) and measles-specific neutralizing antibody levels and IFN- γ ELISPOT responses were evaluated using chi-square tests. We found one replicated association for SNP rs372889 in the *IL12RB1* gene (P=0.03 for Ab^{high}CMI^{high} versus Ab^{low}CMI^{low}). Our findings demonstrate the importance of replicating genotypic-phenotypic associations, which can be achieved using immunophenotypic extremes and smaller sample sizes. We speculate that *IL12RB1* polymorphisms may affect IL-12 and IL-23 binding and downstream effects, which are critical cytokines in the CMI response to measles vaccine.

Keywords

Measles immunity; SNP; Cytokine Receptor; *IL12RB1*; Replication Study

1.1 Introduction

A resurgence of measles outbreaks, along with other vaccine-preventable diseases (i.e., mumps and rubella), have been reported worldwide. In fact, from 2001 to 2008 a median of

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Conflict of Interest

Dr. Poland is the chair of a DMSB for novel non-measles vaccines undergoing clinical studies by Merck Research Laboratories. The other authors do not have any conflicts of interest.

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56 confirmed measles cases was reported annually in the United States, and between January and September 15, 2011, 211 confirmed cases of measles were reported [1]. Eighteen of the 211 cases were in people who had received one or more doses of the MMR (measles/mumps/rubella) vaccine. Hence, primary vaccine failure and waning immunity limit protection against the disease in certain vaccinated individuals. This is attributed in large part to inter-individual genetic variation of immune responses [2,3,4].

Innate and adaptive measles immune responses are genetically influenced as evidenced by the heritability of measles antibody response of 89% [2]. Specific polymorphisms of cytokines and their receptors have been shown to alter cytokine levels and/or activity, impacting cytokine function and potentially, the overall balance of Type I helper T cell (Th1)/Th2/Th17 cytokines [5]. Cytokines and their receptors are essential to measles-induced immunity following either contact with the wild-type viral strain (infection) or the live Edmonston-Enders viral vaccine strain [6,7]. Therefore, cytokine and cytokine receptor gene polymorphisms, especially in coding and promoter regions, may increase (or decrease) the one's susceptibility to measles infection and/or their response to measles vaccine.

Identification of associations between cytokine and cytokine receptor gene polymorphisms and variations in humoral and CMI responses provides information regarding the use of cytokines as markers of immune response [8] or as potential adjuvants [9] in new measles vaccines. Replication of these associations, using an independent population from the discovery study, is necessary to differentiate true genetic associations from Type I statistical errors [10].

While large sample sizes are typically required [11] for association studies, sufficient power can be attained with a substantially smaller sample size by selectively genotyping individuals with phenotypic immune "extremes" [12]. The objective of this study was to validate previously reported discovery associations between cytokine and cytokine receptor SNPs and humoral and CMI responses using a subcohort of children and young adults with extreme humoral and cell-mediated immune responses.

1.2 Materials and Methods

1.2.1 Subjects

Briefly, study subjects from a preceding discovery [13] and current replication study were selected from two independent cohorts of previously recruited, randomly selected healthy children and young adults from all socioeconomic backgrounds in Olmsted County, MN, who had been immunized with two doses of MMR vaccine. The discovery cohort included 346 eligible subjects between 12 to 18 years of age (93% Caucasians), and the current replication cohort included 758 separate, eligible subjects between 11 to 22 years of age (91% Caucasians). No known wild-type measles virus had been reported within the geographical area for each of the subject's lifetime. All human studies were approved by the institutional review board of the Mayo Clinic and informed consent was obtained. Further details regarding study enrollment can be found elsewhere for the original discovery [14] and replication study cohorts [15].

Measles vaccine-specific humoral and CMI responses were quantified by a measles-specific IgG enzyme immunoassay (Dade Behring, Marburg, Germany) [17] and a lymphoproliferation assay (SI 3 indicates positive response) [13] (respectively) for the 346 subjects in the discovery cohort; and a measles virus plaque reduction neutralization (PRN) assay [16,17] and an IFN- γ enzyme-linked immunosorbent spot (ELISPOT) assay [18] (respectively) for the 758 subjects in the replication cohort. These methods reflect

differences in gold-standard methods, at the time, for measuring humoral and CMI responses in each cohort.

The PRN assay was performed using serial dilution replicates of heat-inactivated sera samples and standards (3rd WHO international anti-measles antibody standard). The GFP-positive plaques were scanned and counted using high-throughput fluorescent microscopy; and their titers were transformed to mIU/ml units using the 3rd WHO standard. A neutralizing antibody titer of ≥ 210 mIU/ml, corresponding to a PRN titer of 120 mIU/ml [16], was considered indicative of seroprotection. Low variability (CV=5.7%) was found based the standard values, which also served as a part of the quality assurance/quality control efforts. Further details are described elsewhere [16,17].

For the IFN- γ ELISPOT assay, subjects' peripheral blood mononuclear cells (PBMC) were stimulated with measles vaccine virus (MOI=0.05; 42 hr incubation) and plated in triplicates onto commercially available kits (R&D Systems, Minneapolis, MN). Positive (stimulation with PHA) and negative (un-stimulated) controls were used for each subject. Further details are as previously described [18].

In our initial discovery study [13] and the current replication study, a sub-cohort of subjects with immunophenotypic extremes based on humoral and cell-mediated immune response to measles vaccine were identified. For each cohort, scatterplots of log-transformed humoral and cell-mediated immune response measures were generated. A two-step quadrant selection approach based on these scatterplots was performed to identify subjects falling into one of four "extreme" adaptive immune response quadrants: $Ab^{low}CMI^{low}$, $Ab^{low}CMI^{high}$, $Ab^{high}CMI^{low}$ and $Ab^{high}CMI^{high}$. First, subjects with either humoral or cell-mediated immune response values falling within the middle 50% of all values were removed from the sampling pool. Second, of the remaining subjects, the squared distance from each of the transformed variables and the intersection of the horizontal and vertical median line within the corresponding quadrant was calculated. The 30 subjects with the largest squared distances in each quadrant were characterized as phenotypic "extremes" and were selected as the sub-cohorts for use in this study. It is worth mentioning that the final sub-cohort sample size selected for the preceding discovery study (1st cohort) was 118 subjects (n=29–30 per quadrant) because two subjects did not provide informed consent to be tested in subsequent vaccine-related studies. The final sub-cohort sample size selected for the current replication study (2nd cohort) was 120 subjects (n=30 per quadrant). Subject selection using this quadrant approach is depicted in Figure 1.

1.2.2 SNP genotyping

Fifty-eight tag SNPs in candidate genes were originally selected for genotyping in the phenotypic extremes of the 1st cohort. Selection criteria consisted of a minor allele frequency of ≥ 0.05 located within a particular loci (coding region or 5' or 3' untranslated region or intron), and a previous genotype:phenotype association. Further details have been previously published [13]. Prior to genotyping, genomic DNA was first extracted from clotted blood with the Puregene extraction kit (Gentra Systems, Minneapolis, MN) and DNA concentrations were measured using the Picogreen method (Molecular Probes, Carlsbad, CA) for the discovery and the current studies. Custom Illumina GoldenGate SNP panels were used for the 768 total SNPs. Quality control measures included replicates of genomic DNA sequence standards (e.g., CEPH standards from Coriell), which were used to refine clustering, and replicates without DNA. In addition, subjects with low call rate SNPs (<0.95) were removed from analysis.

For the current study, seven SNPs were successfully genotyped and analyzed using custom Illumina Goldengate SNP panels, as described previously [14,15]. While 11 cytokine and

cytokine receptor gene SNPs (out of 58) in the 1st cohort had a significant association with humoral and cell-mediated immune responses among all four quadrants and/or among Ab^{low}CMI^{low} vs. Ab^{high}CMI^{high}, four SNPs were not included in this replication study due to either high linkage disequilibrium or genotyping failures. Two SNPs in the *IL10* gene (rs1800871 and rs1800872), along with two SNPs in the *IL12B* (p40 subunit) gene (rs2421047 and rs3212227), were in linkage disequilibrium ($r^2=1.0$). Therefore, only one SNP in each gene (rs3212227 and rs1800871) required genotyping. Furthermore, two SNPs (rs2069763 in *IL2* gene and rs3213093 in *IL12* (p40 subunit) gene) failed genotyping and thus were not included in the analyses.

1.2.3 Statistical analyses

For analyses comparing the 30 subjects in the Ab^{low}CMI^{low} group to the 30 in the Ab^{high}CMI^{high} group, assuming an ordinal (log-additive) genotypic effect and two-sided test of hypothesis with Type I error rate of 0.05, we would have 80% power to detect odds ratios of 2.85 and 2.66 for SNPs with minor allele frequencies of 0.2 and 0.3, respectively. Power would be somewhat higher for comparisons across all four groups.

In order to examine whether associations between the seven SNPs in cytokine and cytokine receptor genes and the humoral and CMI responses achieved significance within each defined quadrant, chi-square tests were initially used. As with the discovery set analyses, statistical comparisons were made among all four quadrants as well as between the two most extreme phenotypic quadrants (Ab^{low}CMI^{low} vs. Ab^{high}CMI^{high}). Subsequent analyses used multi- categorical logistic regression analysis to account for the potential confounding effects of the following variables: age at first and second MMR vaccinations, age at enrollment, race, and gender. Parameter estimates often differed by more than 10% in models that included these covariates than in those that did not, indicating possible confounding differences in distributions across quadrants. Thus, all replication set results presented are based on these multivariate logistic models. All statistical tests were two sided and were performed using SAS 9.3 software (SAS Institute, Cary, NC). Additional details regarding statistical methods for the discovery set SNPs have been previously described [13].

1.3 Results

The median and IQR for measles-specific responses to neutralizing antibody (humoral) and IFN- γ ELISPOT (CMI) are shown in Table 1 for each phenotypic quadrant. Associations between the four SNPs in the cytokine genes (*IL2* rs2069762; *IL10* rs1800890 and rs1800871; and *IL12B* rs3212227) and measles-specific humoral and CMI responses did not reach statistical significance ($P > 0.05$; data not shown). In contrast, among the three cytokine receptor gene SNPs analyzed, one association was replicated (Table 2) between an intronic SNP in the *IL12RB1* gene and measures of measles vaccine-induced immunity.

1.4 Discussion

Replication studies are necessary to differentiate true from false positives, especially for complex traits like immunity. These studies can be performed economically and efficiently using chi-squared tests on subjects with phenotypic extremes, which for the current study were defined as Ab^{high/low} and CMI^{high/low}. Our objective was to utilize this method to replicate associations between cytokine and cytokine receptor SNPs and humoral and CMI responses. Discovery and replication study design and analyses were similar, with the exception of different, yet comparable, subject cohorts and immunological assays. One out of seven SNP associations was replicated: rs372889 in the *IL12RB1* gene.

IL12RB1 encodes for IL-12R β 1 that binds to the p40 subunits of IL-12 (IL-12B) and IL-23. IL-12 or IL-23 signal transduction occurs when IL-12R β 1 forms a complex with IL-12R β 2 or IL-23R, respectively [19]. Both cytokines are able to stimulate production of IFN- γ and are important in eliminating a wide array of intracellular viral and bacterial pathogens, such as measles [20]. IL-12 is necessary for cell-mediated immunity [21], and IL-23 stimulates Th17 differentiation. Th17 cells are involved in inflammation, autoimmunity, and vaccine-induced immunity [22,23]. Therefore, the genetic polymorphism rs372889 among Ab^{low}CMI^{low} and Ab^{high}CMI^{high} immune responders may impact not only IL-12, but also IL-23 binding and/or binding activity.

In addition to the association between SNP rs372889 and measles-specific humoral and cell-mediated immune responses, we have previously demonstrated that this particular SNP was also associated with higher mumps-specific humoral (P=0.03) and lower mumps-specific CMI (P=0.02) responses following MMR vaccination [24]. In the same study, another intronic SNP in the *IL12RB1* gene (rs1870063) was significantly associated with variations in mumps-specific humoral (P=0.01) and CMI (P=0.01) responses. However, replication studies are still needed to confirm that rs372889 and rs1870063 contribute to variations in mumps-specific immunity.

Interestingly, the two tag SNPs within the *IL12RB1* gene associated with variations in measles and/or mumps are located in intronic regions. Depending on the type of intron and location of the SNP, mis-splicing may occur, leading to shifts in the reading frame (exon skipping) or retention of introns in the mRNA (intron retention) [25]. These mutations may result in protein modification and diversity and possibly, in this case, contribute to measles/mumps vaccine failure [26]. It is also possible that these tag SNPs are not the causal SNPs but are in high linkage disequilibrium with the causal SNP, which may be in a coding region (i.e., non-synonymous SNP). While not the subject of this paper, fine mapping and functional studies will be performed in future studies to define the precise causal variant and to determine the direct effects and/or downstream functional consequences on immune outcomes, respectively.

The strengths of our study include documentation of two MMR doses for all subjects, and similar demographics of subjects within the 1st and 2nd cohorts. The use of selected samples with extreme immunophenotypes versus random population samples maximized variability and statistical power for differentiating true from false positives [12], while being economically parsimonious. A limitation of our study is an increased signal-to-noise ratio, since statistical power may be affected by the use of different humoral and cell-mediated immune response measures in the 1st and 2nd cohorts [27]. Also, the modest sample size of the 1st and 2nd cohorts may have limited our ability to detect smaller genetic effects. For the current study, we feel that the primary reason for spurious associations in the original study was chance. Since the subjects from the discovery and replication studies were recruited in Olmsted County with similar demographics, it is not likely that allelic heterogeneity due to different ethnicities, selection bias, or population stratification led to non-replication.

Our reason for using different measures of humoral and cell-mediated immune responses for the two studies was that the use of newer gold-standard immunoassays in the recently recruited 2nd cohort was more appropriate. It is worth noting that correlations between EIA and neutralizing antibody assays for measurement of humoral immune response and between lymphoproliferation and IFN- γ ELISPOT for measurement of CMI response have been reported to be quantitatively equivalent [17,28]. Furthermore, since the selected population in the 1st and 2nd cohorts consisted of immunophenotypic extremes versus a randomized population, humoral and cell-mediated immune responses, as measured by two different (yet comparable) types of immunologic assays, would be expected to not vary

significantly. However, if variation of the different humoral and/or cell-mediated immunologic assays occurred, our findings of a similar allelic direction of the association for SNP rs372889 is indicative that the association was not assay-sensitive, further corroborating that the association is authentic.

In conclusion, this replication study strengthened our confidence that the previously reported association between *IL12RB1* SNP rs372889 and measles-specific humoral and cell-mediated immune responses is indeed a true positive. This measles-specific association, along with the mumps-specific associations of *IL12RB* SNPs (especially SNP rs372889) previously discovered (although not yet replicated) in our laboratory [24], may be useful to better understand vaccine-induced immunity and/or design new candidate MMR vaccines or directed adjuvants. Because measles, mumps and rubella are co-administered in a single vaccine, use of an adjuvant that boosts the immune response to two or three of the antigens would be clinically advantageous. Two such potential adjuvants could be the cytokines IL-12 and IL-23, which bind to IL-12R β 1. As genetic polymorphisms (including SNP rs372889) impair IL-12R β 1 binding and/or binding activity, increasing the concentrations of its agonists (e.g., IL-12 and IL-23) will increase the concentration of bound IL-12R β 1 and thereby increase the magnitude of effect of IL-12 and IL-23 down-stream signaling cascades. As more information regarding “true” associations between genetic polymorphisms and vaccine-induced immunity are developed, improved viral vaccines may be designed that will one day eradicate contagious and life-threatening viral infections, such as measles [29,30].

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Abbreviations

CMI	cell-mediated immunity
MMR	Measles/mumps/rubella
Ab	Antibody
CDC	Centers for Disease Control and Prevention
Th1	helper T cells

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Fig. 1.

Selection of a set of study subjects based on a measles vaccine-specific humoral (neutralizing antibody levels) and cell-mediated (quantitation of IFN- γ -secreting lymphocytes) immune response. A (●) represents the 30 subjects with the most extreme phenotypes in each quadrant, whereas a (○) represents the remaining subjects not included in the SNP genotyping or statistical analyses. Horizontal and vertical solid lines represent the median lines for the cell-mediated and humoral immune responses (respectively) in each quadrant. Horizontal and vertical dashed lines represent the upper and lower IQR for the cell-mediated and humoral immune responses (respectively) in each quadrant.

Clinical measures of measles-specific humoral (neutralizing Ab) and cell-mediated (IFN- γ ELISPOT) immune responses following 2 doses of MMR vaccination for each phenotypic quadrant.

Table 1

Quadrant	N	Variable	Median mIU/ml	Lower Quartile mIU/ml	Upper Quartile mIU/ml
Ab ^{low} CMI ^{low}	30	Neutralizing Ab	208	149	290
		IFN- γ ELISPOT	6	1	9
Ab ^{low} CMI ^{high}	30	Neutralizing Ab	184	127	329
		IFN- γ ELISPOT	113	91	144
Ab ^{high} CMI ^{low}	30	Neutralizing Ab	2807	2184	3801
		IFN- γ ELISPOT	3	0	7
Ab ^{high} CMI ^{high}	30	Neutralizing Ab	2784	1977	3321
		IFN- γ ELISPOT	100	83	116

Table 2

Cytokine receptor SNPs associated with measles-specific humoral (Ab) and cell-mediated (CMD) immune response extremes following 2 doses of MMR vaccination.

Gene	SNP (variant name)	Genotype	Ab ^{low} CMI ^{low} (N=30)	Ab ^{low} CMI ^{high} (N=30)	Ab ^{high} CMI ^{low} (N=30)	Ab ^{high} CMI ^{high} (N=30)	χ^2 P ^a for all Quadrants	χ^2 P ^a for Ab ^{low} CMI ^{low} vs. Ab ^{high} CMI ^{high}
<i>IL4RA</i>	rs1805010 (Ex5+14C)	AA	6 (20)	11 (37)	7 (23)	6 (20)	0.21	0.09
		GA	15 (50)	11 (37)	17 (57)	20 (67)		
		GG	9 (30)	8 (27)	6 (20)	4 (13)		
<i>IL12RB1</i>	rs372889 (IVS14-517C)	AA	10 (33)	9 (30)	8 (27)	4 (13)	0.12	0.03 ^b
		GA	15 (50)	11 (37)	17 (57)	13 (43)		
		GG	5 (17)	10 (33)	5 (17)	13 (43)		
<i>IL12RB2</i>	rs3790567 (IVS8+5606A)	AA	4 (13)	2 (7)	2 (7)	0 (0)	0.32	0.38
		GA	12 (40)	14 (47)	6 (20)	11 (37)		
		GG	14 (47)	14 (47)	22 (73)	19 (63)		

^aP-values based on multi-categorical logistic regression analysis, adjusted for age at enrollment, gender, race, age at first MMR and age at second MMR

^bStatistically significant association (P 0.05)