HLA homozygosity does not adversely affect measles vaccine-induced cytokine responses

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Abstract

The association between HLA homozygosity and measles-specific Th$_1$ (IFN-γ, IL-2 and IL-12p40) and Th$_2$ (IL-4 and IL-10) cytokine responses were assessed in a group of 339 healthy schoolchildren 12–18 years of age previously immunized with two doses of live-attenuated measles virus vaccine. No associations were observed between class I HLA homozygosity and measles-specific cytokine levels. Children who were homozygous at the class II DRB1, DQA1, DPA1 and DPB1 loci had higher median IFN-γ secretion levels compared with children who were heterozygous for DRB1 (77.7 vs. 39.5 pg/ml, $p=0.05$), DQA1 (60.9 vs. 36.6 pg/ml, $p=0.03$), DPA1 (46.1 vs. 27.1 pg/ml, $p=0.01$) and DPB1 (61.5 vs. 36.0 pg/ml, $p=0.01$) loci, respectively. Homozygosity at increasing numbers of HLA loci ($>=4$) was associated with increased IFN-γ secretion levels (test for trend p-value$=0.01$). Our results suggest that HLA homozygosity showed no disadvantage for measles-specific cytokine responses and instead was associated with increased IFN-γ levels.
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Introduction

Immune responses to measles virus (MV) immunization result from the interaction of the virus and a variety of immune response genes, particularly host human leukocyte antigen (HLA) genes. Given its important role in antigen presentation, polymorphisms in the HLA genes restrict T lymphocyte responses to measles thereby influencing measles vaccine virus-induced immunity.

The genetic basis for the variation in the immune response to viruses, including hepatitis B, influenza, and HIV-1, has been recognized (Kruskall et al., 1992; Gelder et al., 2002; Kaslow et al., 2001; Newport et al., 2004). Specific class I and class II HLA alleles have been associated with the spectrum of immune response to measles vaccine (Poland et al., 2001; St.Sauver et al., 2002; Jacobson et al., 2003; Ovsyannikova et al., 2004b). In particular, children who were homozygous for alleles within classes IB and DQA1 were found more likely to be seronegative than children who were heterozygous at these loci (St.Sauver et al., 2002).

Theoretically, HLA homozygosity – defined as "inheritance of two identical alleles at each polymorphic locus" – may limit the repertoire of immune responses by reducing the diversity of pathogen-derived peptides which can be displayed on the surface of antigen-presenting cells ("the heterozygote advantage") and confers an unfavorable prognosis following infection (Thursz et al., 1997; Tang et al., 1999; Trachtenberg

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et al., 2003; Carrington and O'Brien, 2003). Accordingly, previous studies demonstrated an association between homozygosity, or reduced HLA diversity, and decreased measles-induced antibody levels following a single dose of vaccine (St.Sauver et al., 2002). However, two doses of measles vaccine could overcome this barrier and induced protective antibody levels and lymphoproliferative immune responses regardless of HLA homozygosity status (St.Sauver et al., 2005). Associations between HLA homozygosity and cytokine immune responses to viral vaccines, such as measles, in a genetically diverse population have not been well studied. To address the fundamental question of whether the mechanism of lower vaccine-induced antibody levels in the context of HLA homozygosity were related to low vaccine-induced cytokine levels, we examined the relationship between class I and class II HLA homozygosity and Th_1 (interferon gamma [IFN-γ], interleukin-2 [IL-2], and IL-12p40)/Th_2 (IL-4 and IL-10) cytokine immune responses. The study presented here provided an opportunity to assess for the first time the relative contribution of homozygous HLA genotypes in the control of cytokine immune responses in adolescents.

Results

Study population and measles-specific cytokine responses

In total, 339 children were enrolled in the study ranging in age from 12 to 18 years. The majority of the children were white (93%) and the median age of participants at the first and second immunization was 15.6 months and 12.1 years, respectively. The median time from second immunization to blood draw was 4.7 years. There were a total of 159 (47%) girls and 180 (53%) boys in the study. IFN-γ, IL-4, and IL-10 cytokine responses to MV within the total population were similar between genders (median IFN-γ secretion of 49.7 pg/ml for girls versus 31.4 pg/ml for boys [$p=0.39$], median IL-4 secretion of 9.3 pg/ml for girls versus 10.0 pg/ml for boys [$p=0.72$], and median IL-10 secretion of 32.0 pg/ml for girls versus 24.5 pg/ml for boys [$p=0.19$], respectively). IL-2 cytokine responses were below the minimum detection limit (median IL-2 secretion of -1.9 pg/ml for girls versus -2.8 pg/ml for boys [$p=0.51$]). Examination of measles-specific IL-12p40 cytokine responses revealed that girls had marginally higher IL-12p40 cytokine responses than boys (median 9.0 pg/ml for girls versus 6.5 pg/ml for boys, respectively [$p=0.07$]). Rates of class I A, B, Cw, and class II DRB1, DQA1, DQB1, or DPB1 homozygosity did not differ significantly by gender. However, DPA1 homozygosity was significantly different between genders: 111 (64%) of the boys and 115 (75%) of the girls were homozygous at the DPA1 locus ($p=0.03$) (Table 1). We also found that specific A∗02, B∗44, Cw∗07, DRB1∗15/16, DQA1∗01, DQB1∗03, DPA1∗01, and DPB1∗04 alleles accounted for 48%, 22%, 56%, 25%, 62%, 37%, 91%, and 88% of the A, B, Cw, DRB1, DQA1, DQB1, DPA1 and DPB1 alleles, respectively. No violations of Hardy–Weinberg equilibrium were found for

Table 1
Comparison of locus-specific homozygosity rates across gender

HLA locus	Number of homozygous males (%)	Number of homozygous females (%)	P-value [a]
HLA-A	42 (23.3)	31 (19.5)	0.39
HLA-B	17 (9.4)	10 (6.3)	0.28
HLA-Cw	35 (19.4)	20 (12.6)	0.09
HLA-DRB1	26 (14.4)	18 (11.3)	0.39
HLA-DQA1	49 (28.2)	38 (24.5)	0.45
HLA-DQB1	63 (35.0)	55 (34.6)	0.93
HLA-DPA1	111 (63.8)	115 (74.7)	**0.03**
HLA-DPB1	47 (26.1)	53 (33.3)	0.14

[a] P-value compares distributions of homozygosity across gender using χ^2 tests of significance. Results significant at a p-value ≤ 0.05 are highlighted in bold.

HLA-A ($p=0.38$), B ($p=0.91$), DRB1 ($p=0.11$), DQA1 ($p=0.09$), DPA1 ($p=0.12$), or DPB1 ($p=0.61$) loci. However, a comparison of allele distributions for the HLA-Cw and HLA-DQB1 loci revealed possible departures from equilibrium ($p=0.03$ and $p<0.001$, respectively). As a result, statistical comparisons involving the Cw and DQB1 loci should be viewed with caution.

HLA homozygosity and cytokine immune responses

No associations were observed between HLA class I A, B or Cw homozygosity and measles-specific Th_1-like (IFN-γ, IL-2, and IL-12p40) cytokine levels ($p=0.11$ to 0.95) (Table 2). However, significant associations with class II loci were observed for IFN-γ responses following measles vaccination. Children who were homozygous at class II DRB1, DQA1, DPA1 and DPB1 loci had significantly higher median IFN-γ secretion levels compared with children who were heterozygous for DRB1 (77.7 vs. 39.5 pg/ml, $p=0.05$), DQA1 (60.9 vs. 36.6 pg/ml, $p=0.03$), DPA1 (46.1 vs. 27.1 pg/ml, $p=0.01$) and DPB1 (61.5 vs. 36.0 pg/ml, $p=0.01$) loci, respectively (Table 2). Measles-specific IL-2 cytokine immune responses were below minimum detectable value. Measles-induced IL-12p40 cytokine immune responses were low and demonstrated no evidence of genetic regulation by any of the loci considered ($p=0.14$ to 0.88). Since the DPA1 locus was not very polymorphic in our population (DPA1∗0103 and DPA1∗0201 accounted for 77% and 17% of the DPA1 alleles, respectively) and 226 (70%) of the children in the study were homozygous at this locus, we excluded DPA1 in the overall homozygosity analyses.

The relationship between HLA homozygosity and measles-induced Th_2-like (IL-4 and IL-10) cytokine immune responses was also examined. The median IL-4 secretion levels among children who were homozygous at the class IA locus was marginally significantly different from the median IL-4 levels among children who were heterozygous at this locus (9.1 vs. 10.3 pg/ml, $p=0.07$) (Table 3). Children who were homozygous at the DQA1 locus had suggestive evidence of higher median measles-specific IL-10 levels compared with children who were heterozygous for DQA1 (32.7 vs. 26.0 pg/ml, $p=0.06$). However, multilocus homozygosity was not associated with

Table 2
Differences in distribution of measles-specific IFN-γ, IL-2 and IL-12p40 responses between children heterozygous and homozygous for specific HLA loci

HLA locus	IFN-γ secretion (pg/ml)				IL-2 secretion (pg/ml)				IL-12p40 secretion (pg/ml)			
	Homozygotes, N	Heterozygotes, median (IQR)	Homozygotes, median (IQR)	p-value[a]	Homozygotes, N	Heterozygotes, median (IQR)	Homozygotes, median (IQR)	p-value[a]	Homozygotes, N	Heterozygotes, median (IQR)	Homozygotes, median (IQR)	p-value[a]
A	73	40.76 (6.88, 195.79)	40.59 (13.46, 109.92)	0.81	73	−2.84 (−15.59, 14.70)	0.65 (−10.50, 11.39)	0.65	73	8.81 (3.0, 18.0)	6.0 (2.0, 14.0)	0.14
B	27	43.51 (9.66, 189.07)	9.78 (−1.37, 97.17)	0.11	27	−2.27 (−15.25, 13.56)	−2.44 (−10.15, 15.89)	0.74	27	8.0 (2.04, 18.0)	5.0 (1.96, 9.0)	0.19
Cw	55	40.84 (8.94, 189.07)	38.79 (1.17, 115.65)	0.95	55	−3.21 (−14.88, 13.17)	3.07 (−15.59, 15.89)	0.58	55	8.0 (2.0, 16.0)	8.0 (3.0, 19.0)	0.58
DRB1	44	39.57 (7.92, 167.11)	77.77 (11.68, 329.28)	**0.05**	43	−1.94 (−15.25, 13.19)	−4.22 (−14.53, 15.89)	0.97	44	8.0 (2.0, 17.5)	7.0 (1.5, 15.0)	0.88
DQA1	87	36.69 (6.88, 154.13)	60.98 (13.46, 226.8)	**0.03**	86	−2.86 (−15.42, 13.7)	0.32 (−14.72, 14.7)	0.62	87	7.0 (2.0, 17.0)	9.0 (1.0, 18.0)	0.50
DQB1	118	38.79 (9.0, 148.81)	48.74 (7.92, 264.28)	0.27	116	−2.77 (−15.42, 13.7)	−1.78 (−14.52, 14.62)	0.53	118	8.0 (2.0, 16.5)	8.08 (2.04, 18.00)	0.55
DPA1	226	27.19 (3.72, 97.46)	46.18 (9.79, 205.73)	**0.01**	224	−0.8 (−12.78, 17.74)	−2.83 (−15.43, 13.39)	0.11	225	7.5 (3.0, 13.0)	8.0 (2.0, 18.0)	0.70
DPB1	100	36.03 (5.67, 133.48)	61.52 (13.52, 247.59)	**0.01**	100	−1.76 (−14.85, 14.54)	−3.63 (−15.13, 13.18)	0.30	100	7.0 (2.0, 15.67)	9.92 (3.5, 19.0)	0.15
Any locus[b]	259	34.91 (2.74, 120.84)	46.29 (9.64, 211.75)	0.13	257	−4.03 (−17.16, 12.21)	−1.81 (−14.53, 13.89)	0.48	259	7.0 (2.0, 13.0)	8.0 (2.0, 18.0)	0.52

[a] Linear regression analysis. P-values were calculated using rank-transformed values; p-values adjusted for age at enrollment, age at first MMR vaccination, age at second MMR vaccination, gender, and race. Results significant at a p-value ≤ 0.05 are highlighted in bold.
[b] Analysis does not include DPA1.

Table 3
Differences in distribution of measles-specific IL-4 and IL-10 responses between children heterozygous and homozygous for specific HLA loci

HLA locus	IL-4 secretion (pg/ml)				IL-10 secretion (pg/ml)			
	Homozygote, N	Heterozygote, median (IQR)	Homozygote, median (IQR)	p-value[a]	Homozygote, N	Heterozygote, median (IQR)	Homozygote, median (IQR)	p-value[a]
A	73	10.38 (4.29, 24.82)	9.1 (0.0, 21.75)	0.07	73	28.5 (10.0, 72.5)	29.5 (11.0, 74.5)	0.78
B	27	9.65 (2.53, 24.37)	10.86 (2.8, 22.51)	0.99	27	29.5 (10.0, 75.5)	24.0 (14.5, 41.5)	0.47
Cw	55	9.65 (2.12, 24.63)	10.86 (4.91, 23.11)	0.65	55	29.5 (11.5, 74.5)	25.0 (−4.0, 52.0)	0.24
DRB1	44	9.85 (2.3, 24.82)	9.22 (4.35, 21.17)	0.69	43	28.5 (10.0, 74.5)	32.5 (13.0, 70.0)	0.30
DQA1	87	9.59 (2.05, 23.85)	9.55 (4.29, 23.88)	0.55	86	26.0 (7.75, 69.75)	32.75 (14.0, 84.5)	0.06
DQB1	118	10.29 (2.95, 24.2)	8.54 (2.22, 24.82)	0.57	116	26.0 (8.5, 78.25)	32.75 (12.5, 66.0)	0.87
DPA1	226	9.14 (1.02, 19.58)	9.92 (4.19, 25.34)	0.77	224	22.5 (10.0, 47.0)	32.75 (10.75, 78.0)	0.17
DPB1	100	10.07 (3.99, 25.65)	8.73 (0.92, 20.69)	0.16	100	29.0 (10.25, 75.5)	28.0 (8.0, 69.5)	0.49
Any locus[b]	259	10.02 (4.06, 25.57)	9.59 (2.19, 23.11)	0.75	257	25.5 (9.5, 81.5)	32.0 (10.5, 71.0)	0.93

[a] Linear regression analysis. *P*-values were calculated using rank-transformed values; *p*-values adjusted for age at enrollment, age at first MMR vaccination, age at second MMR vaccination, gender, and race.

[b] Analysis does not include DPA1.

Table 4
Differences in distribution of measles-specific cytokine responses among children homozygous for increasing numbers of HLA loci

Number of homozygous loci	N	Median cytokine secretion values (IQR), pg/ml	*P*-value[a]
IFN-γ secretion			
0	42	22.32 (0.49, 88.29)	**0.01**
1	76	42.53 (9.39, 161.45)	
2	100	40.46 (7.10, 123.30)	
3	61	57.74 (15.31, 277.78)	
≥4	60	56.67 (3.90, 229.90)	
IL-2 secretion			
0	42	−1.36 (−12.12, 12.21)	1.00
1	75	−4.16 (−16.19, 17.74)	
2	99	−2.84 (−14.88, 13.80)	
3	60	−3.88 (−15.16, 8.40)	
≥4	60	3.66 (−12.78, 18.31)	
IL-12p40 secretion			
0	42	7.00 (3.00, 11.00)	0.76
1	75	9.00 (2.00, 18.00)	
2	100	7.00 (2.00, 17.00)	
3	61	9.83 (3.00, 18.00)	
≥4	60	7.08 (3.00, 17.00)	
IL-4 secretion			
0	42	7.37 (1.22, 19.58)	0.54
1	76	11.56 (4.84, 27.34)	
2	100	11.12 (2.26, 25.65)	
3	61	10.48 (4.52, 25.34)	
≥4	60	7.43 (1.50, 20.22)	
IL-10 secretion			
0	42	20.25 (10.00, 37.00)	0.62
1	75	30.50 (6.50, 86.50)	
2	99	36.00 (6.00, 78.50)	
3	60	32.25 (14.00, 72.00)	
≥4	60	27.00 (10.25, 55.25)	

[a] Linear regression analysis test for trend; calculated using rank-transformed values; *p*-values adjusted for age at enrollment, age at first MMR vaccination, age at second MMR vaccination, gender, and race. Results significant at a *p*-value ≤ 0.05 are highlighted in bold.

measles-induced IL-4 and IL-10 secretion levels ($p = 0.75$ and 0.93, respectively).

Finally, we examined the associations between homozygosity at increasing number of HLA loci (including DPA1) and measles-induced cytokine secretion levels. Homozygosity at increasing numbers of HLA loci (≥4) was associated with increased IFN-γ secretion levels (test for trend *p*-value = 0.01) (Table 4). Homozygosity at increasing numbers of HLA loci was not, however, associated with differences in IL-2, IL-12p40, IL-4 or IL-10 secretion levels. All analyses described above defined homozygosity based on the two-digit molecular designation. We re-assessed associations using the four-digit designation and found similar associations with homozygosity, albeit with fewer individuals classified as homozygous.

Discussion

We found little evidence that either homozygosity at specific HLA loci or overall homozygosity had any disadvantage in terms of measles vaccine-induced cytokine immune responses after two doses of measles vaccine. If anything, our study suggests that class II HLA homozygosity is associated with increased IFN-γ levels following two doses of measles vaccine. Moreover, we found that homozygosity at increasing numbers of HLA loci was associated with increased IFN-γ secretion levels, signifying the role of HLA molecules in the control of immune responses to MV. This may indicate that IFN-γ is important for immunity to measles; however, it is unknown how homozygosity and polymorphic differences within HLA loci affect peptide binding, T lymphocyte recognition, and hence the outcome of IFN-γ production. It is clear that more work needs to be done to elucidate the exact mechanism for increased IFN-γ levels in HLA homozygous individuals. Our results lend support to studies that indicate that IFN-γ and IL-4 responses to MV are mediated primarily by CD4$^+$ T cells with a Th$_1$-like phenotype and that depletion of CD4 cells prior to measles stimulation completely abrogated IFN-γ and IL-4 production (Howe et al., 2005a). Other studies have suggested that upon secondary *in vitro* measles stimulation, memory T cells generate

cells that produce either IFN-γ alone or secreted both IFN-γ and IL-4 and resemble Th_1/Th_0-like cells (Howe et al., 2005b). We also found that children who were homozygous at the DQA1 locus had marginally higher measles-specific IL-10 levels compared with children who were heterozygous for DQA1. A different pattern was observed for class I HLA homozygosity. With one exception, there was no significant association between class I HLA homozygosity and cytokine secretion levels after two doses of measles vaccine. Children who were homozygous at the class IA locus had marginally lower IL-4 secreted levels compared with children who were heterozygous at this locus. Furthermore, neither single nor multilocus homozygosity was associated with measles-induced low IL-2 and IL-12p40 secretion levels. The observed low IL-2 and IL-12p40 secretion levels may indicate that MV infection suppresses IL-2 and IL-12 *in vitro* production by peripheral blood mononuclear cells (PBMC). Additionally, multilocus homozygosity was not associated with measles-induced IL-4 and IL-10 secretion levels.

It has been speculated that the lack of any effect of HLA class I homozygosity may reflect lesser contributions of individual polymorphic class I alleles in cytokine immune responses to measles (Tang et al., 1999). In any event, the variable binding affinities to polymorphic HLA molecules and polymorphisms in effector molecules, such as cytokines and other co-stimulatory molecules, may play a role in the immune response to vaccines (Wang et al., 2004; Schuenke et al., 1998; Lindemann et al., 2002). We examined associations of eight HLA loci with each of five different cytokine secretion values, resulting in a total of 40 locus-specific tests of homozygosity; thus, multiple testing issues exist. We found four statistically significant associations, about twice as many as we would expect by chance, and all four involved IFN-γ cytokine secretion. In each, homozygosity was further associated with increased IFN-γ secretion levels.

The outcome of this study to some extent is in agreement with our earlier study demonstrating that a two-dose vaccination regimen may overcome the barrier of HLA homozygosity on single dose measles vaccine-induced low antibody levels (St. Sauver et al., 2005). This extinction of the so-called HLA homozygote disadvantage is an interesting finding. The lack of HLA diversity might reduce the repertoire of naturally processed viral epitopes that can be presented to T helper or T cytotoxic lymphocytes, resulting in a decreased immune response to viral antigens (Carrington and O'Brien, 2003). However, we found no statistical support for a heterozygous advantage based on HLA class I and class II loci and cytokine immune responses to measles vaccine. Our results suggest that a two-dose vaccination schedule seems to induce measurable measles-specific cytokine immune responses, despite HLA homozygosity status. As we have observed in a previous study, measles antibody levels following two doses of MMR vaccine were correlated with lymphoproliferation ($r=0.12$, $p=0.03$), but lacked any correlation with the measures of IFN-γ and IL-4 secretion. Alternatively, a significant correlation was found between lymphoproliferation and IFN-γ ($r=0.20$, $p=0.0002$) and IL-4 ($r=0.15$, $p=0.005$) secretion (Dhiman et al., 2005).

Various combinations of polymorphic HLA molecules are involved in shaping the course of immune responses to measles, mainly by controlling humoral and cellular immune responses to the virus (Tang et al., 1999). In this context, Kaslow et al. (2001) demonstrated that class I HLA homozygosity was not significantly disadvantageous when analyzed for response to ALVAC-HIV recombinant canarypox vaccine. In the case of hepatitis B virus (HBV) vaccination, there was overrepresentation of homozygotes for HLA-A1, HLA-B8, HLA-DR3 and HLA-DQ2 alleles in non-responders to this vaccine (Vingerhoets et al., 1995; Stachowski et al., 1995). Early papers found evidence that PBMC from HBV non-responders did not produce Th_1 (IL-2 and IFN-γ) cytokines after hepatitis B surface antigen (HBsAg) stimulation (Vingerhoets et al., 1994). To our knowledge, the relationship between HLA homozygosity and secreted cytokines has not been previously studied for measles. We cannot, however, evaluate the relative contributions of individual HLA molecules to cytokine production following measles immunization. We speculate that homozygotes for specific HLA alleles of the DRB1, DQA1, DPA1 or DPB1 loci could present a similar or broader range of MV-derived peptides compared to certain heterozygotes (Tang et al., 1999). In fact, peptides that differ in sequence but share multiple key amino acid motifs capable of fitting into key HLA anchoring pockets may be bound by the same HLA molecule ("peptide promiscuity") (Hill et al., 1994; O'Sullivan et al., 1991; Sidney et al., 1995; Marshall et al., 1994). Studies have demonstrated that different peptides can be bound by the same HLA molecule (Hughes et al., 1996; Yassine-Diab et al., 1999) and each HLA allele "binds an overlapping but distinct set of peptides, with a resultant unique potential immune repertoire," (Nepom et al., 1996) and has "a readily distinguishable and reproducible peptide profile" (Chicz et al., 1993). Similarly, recognition of viral peptides with "dual" specificity (for example, binding to both class IA and DRB1 alleles), which elicit both HLA class I- and class II-restricted immune responses, can add to the complexity of our findings (Sette et al., 1991). On the other hand, no significant heritability was observed for the IFN-γ responses to the naturally derived immunodominant epitope from the *Mycobacterium tuberculosis* Ag85B that can be presented in a promiscuous manner by multiple HLA class II molecules encoded by the *DRB1*, *DRB3*, and *DRB4* genes (Mustafa et al., 2000). Nonetheless, the existence of HLA class I and II supertypes (Sette and Sidney, 1999; Sidney et al., 1996; Ou et al., 1998) and the description of HLA alleles capable of sharing overlapping peptide-binding specificities (Sidney et al., 1995; Southwood et al., 1998), could facilitate our understanding of the genetic effect of HLA homozygosity on vaccine immune response.

Limitations of our study include lack of data on the *in vitro* cytokine response profile and correlation with antibody titers in the vaccine recipients. In addition, measles-specific cytokine production can be transient and any differences can be obscured by using cell culture supernatants from frozen PBMC. Nonetheless, results from our population-based study suggest that

HLA homozygosity showed no disadvantage for measles vaccine-induced cytokine responses and instead was associated with increased IFN-γ levels. Further studies are required to answer the question whether HLA homozygosity may affect vaccine-generated CD8 and CD4 cytotoxic T lymphocyte response against MV. It is important that our results be validated in a larger study population, since confirmation that HLA homozygosity is not disadvantageous may simplify novel vaccine development.

In conclusion, our results suggest that homozygosity at class I and class II loci showed no disadvantage for measles vaccine-induced cytokine immune responses following two doses of the MMR vaccine. Therefore, live measles vaccine is an effective vaccine inducing measurable cytokine immune responses after two doses of the vaccine, in spite of HLA homozygosity status. Further studies should clarify whether these observations are generalizable to other viral vaccines, such as mumps and rubella. A better characterization of such host genetic profile markers that influence the outcome of vaccine immune responses will support further vaccine development and application.

Materials and methods

Study subjects

Details of our subject recruitment have been published elsewhere (Ovsyannikova et al., 2004a; St.Sauver et al., 2005, in press). In brief, we enrolled 346 healthy children (age 12 to 18 years) identified through the Minnesota Independent School District 535 registration rolls. HLA and cytokine secretion data were available on 339 subjects. Measles vaccination was part of a routine program and all enrolled participants had documentation in their medical records of having received two doses of live measles–mumps–rubella (MMR) vaccine (Merck Research, West Point, PA) containing the attenuated Edmonston B strain of MV (≥ 1000 TCID$_{50}$). Mayo Clinic's Institutional Review Board granted approval for the study, and blood samples were drawn after informed consent, permission, and assent were obtained as appropriate from the subjects and their parents.

In vitro IFN-γ, IL-2, IL-4, IL-10 and IL-12p40 cytokine responses to measles

Cytokine responses to MV were measured as described previously (Ovsyannikova et al., 2005). Briefly, PBMC were separated from heparinized venous blood by Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. Cells were resuspended in RPMI 1640 (Celox Laboratories Inc., St. Paul, MN) freezing media containing 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and 20% heat-inactivated fetal calf serum (FCS), frozen at -80 °C, and stored in liquid nitrogen. For IFN-γ and IL-12p40 cytokine determination, PBMC were cultured at a concentration of 2×10^5 cells/well in triplicate with either media or Edmonston B vaccine strain of MV (virus stock of 1×10^7 plaque-forming units [pfu]/ml) diluted in RPMI 1640 supplemented with 1% normal human serum (NHS) at a

multiplicity of infection (MOI) of 0.5. In optimization experiments no differences were observed in secreted cytokine responses in the control cell cultures in the presence of RPMI 1640 media versus equivalent Vero cell lysate. Cytokine IL-12p40 was selected since the decrease in IL-12 production by monocytes was shown to be greater when the p40 monomer was measured rather than the bioreactive p70 heterodimer (Karp et al., 1996; Polack et al., 2002). For IL-4 determination, cells were cultured at a concentration of 4×10^5 cells/well in duplicate in the presence of 2 μg/ml of monoclonal IL-4 receptor antibody (mAb) (R&D Systems, Minneapolis, MN) with or without MV diluted in RPMI 1640 supplemented with 1% NHS at a MOI of 0.1 as previously described (Dhiman et al., 2004). For determination of secreted IL-2 and IL-10, PBMC (4×10^5 cells/well) were cultured in duplicate with or without MV (MOI of 0.1). Stimulation with 5 μg/ml phytohemagglutinin (PHA) was used as a positive control in selected subjects. Cell-free supernatants were collected on day 6 and MV-specific IFN-γ, IL-2, IL-4, IL-10, and IL-12p40 responses were quantitatively determined by ELISA following the manufacturer's protocol (BD Pharmingen, San Diego, CA). The levels of sensitivity for the IFN-γ, IL-2, IL-4, IL-10, and IL-12p40 assays were 4.7 pg/ml, 4 pg/ml, 7.8 pg/ml, 4 pg/ml, and 4 pg/ml, respectively. Median background levels of IFN-γ, IL-2, IL-4, IL-10, and IL-12p40 cytokine production in cultures not stimulated with MV were subtracted from the median measles-induced responses to produce corrected secretion values. Negative corrected values indicate that the unstimulated secretion levels were, on average, higher than the stimulated secretion levels.

DNA extraction and HLA typing

Details of HLA typing have been published elsewhere (Ovsyannikova et al., 2004a). High-molecular-weight genomic DNA was extracted from blood samples using a commercial Puregene DNA extraction kit (Gentra Systems Inc., Minneapolis, MN). High-resolution polymerase chain reaction with sequence-specific primer (PCR–SSP) was performed for HLA class I and class II typing using SSP UniTray typing kits (Dynal Biotech, Brown Deer, WI). Ambiguous allele combinations were resolved by utilizing DNA sequencing kits and/or by utilizing a group-specific primary (AmbiSolv) amplification approach. All PCR performed with negative controls and every 50th PCR reaction was repeated for quality control. Homozygosity was declared for individuals when they carried two copies of the same allele, using the four-digit molecular designation of HLA alleles.

Statistical analysis

Five outcomes, in two major classes of cytokine responses to MV, were of primary interest: Th$_1$ (IFN-γ, IL-2, and IL-12p40) and Th$_2$ (IL-4 and IL-10). Levels of each of these cytokines were measured in supernatants in units of picograms per milliliter. Data were descriptively summarized using frequencies and percentages for all categorical variables and medians and inter-quartile ranges for all continuous variables.

Associations of cytokine response with gender were assessed using analysis of variance methods. Due to data skewness, all *p*-values were calculated using rank-transformed values. Associations of homozygosity with gender were assessed using Chi-square tests of significance.

We compared levels of cytokine secretion in homozygous versus heterozygous individuals using linear regression techniques. Variables representing locus-specific homozygosity status were created for each of the eight available HLA loci. We first fit separate models for each of the loci individually. We then created a variable indicating whether a subject was homozygous for at least one of the loci and assessed its relationship with cytokine secretion. The DPA1 locus was not considered in this latter assessment due to its high degree of homozygosity. We then calculated a homozygosity count for each subject. Values of this count could range from 0 to 8, depending on the number of loci for which the subject was homozygous. However, because of sparseness of data, individuals homozygous for five or more loci were grouped with those homozygous for four loci. We used this count variable to assess the possible dose–response relationship between homozygosity and cytokine secretion by fitting the count as a one degree-of-freedom ordinal variable. Again because of data skewness, all *p*-values were calculated using rank-transformed values. All analyses accounted for the effects of the following set of potential confounding variables by including each as a covariate in the linear regression models: age at study enrollment, gender, age at first MMR vaccination, age at second MMR vaccination, and race. Finally, the statistical tests described above assume that the two alleles from each subject are independent; that is, genotype proportions fit Hardy–Weinberg proportions. We tested this assumption using the software HWE (Guo and Thompson, 1992). All statistical tests were two-sided, and all analyses were carried out using the SAS software system (SAS Institute, Inc., Cary, NC).

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