MAJOR ARTICLE

Human Leukocyte Antigen Genotypes in the Genetic Control of Adaptive Immune Responses to Smallpox Vaccine

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Background. The role of human leukocyte antigen (HLA) genes in mediating adaptive immune responses to smallpox vaccine remains unknown.

Methods. We determined genotypes for a group of individuals ($n = 1071$) who received a single dose of smallpox vaccine (Dryvax, Wyeth Laboratories) and examined associations between HLA alleles and 15 immune outcomes to smallpox vaccine on a per-locus and a per-allele level.

Results. We found significant associations between the HLA-B and HLA - DQB1 loci and vaccinia-induced antibodies ($P = .04$ for each locus), with the HLA-B*1302 ($P = .036$), B*3802 ($P = .011$), DQB1*0302 ($P = .015$), and DQB1*0604 ($P = .017$) alleles being associated with higher levels. Significant global associations were identified between vaccinia-specific interferon (IFN)–γ and DQA1 ($P = .003$), interleukin (IL)–1β and HLA-B ($P = .004$), tumor necrosis factor (TNF)–α and HLA-B ($P = .006$), and IL-6 and HLA-B locus ($P = .016$) for secreted cytokines, as well as between $CD8\alpha^+$ IFN-γ Elispot responses and DQB1 ($P = .027$). Subjects carrying B*3906 ($P = .006$) and B*5701 ($P < .001$) secreted higher levels of IL-1β than did subjects who did not carry these alleles. Subjects carrying the B*5301 ($P = .047$) and B*5601 ($P = .008$) alleles secreted less IL-1β, compared with subjects who did not carry these alleles. The B*3502 ($P = .009$), B*5601 ($P = .004$), and B*5701 ($P < .001$) alleles were significantly associated with variations in TNF-α secretion.

Conclusions. These data suggest that variations in antibody and cellular IFN-γ, IL-1β, TNF-α, and IL-6 immune responses after receipt of smallpox vaccine are genetically controlled by HLA genes or genes in close linkage disequilibrium to these alleles.

The impact of human leukocyte antigen (HLA) genes in controlling adaptive immune responses to smallpox vaccine is unknown. Smallpox (variola virus) is a devastating pathogen to humans, causing significant morbidity and mortality, and is a potential agent of bioterrorism [1]. Vaccination with vaccinia virus (VACV) protects against smallpox and related orthopox viruses and results in long-lasting immunity [2]. Smallpox vaccine generates humoral and cellular immunity, but the biological basis for variations in immunity is not well understood [3–5].

Genes play an important role in the host immune response to vaccination in the population. Because HLA gene polymorphisms lead to differential VACV antigen presentation, we speculated that variations in immune response to smallpox vaccine are influenced by polymorphisms of HLA genes. We previously demonstrated associations between HLA polymorphisms and variations in both humoral and cell-mediated immune responses to other viral vaccines, such as measles, rubella, mumps, and influenza [6–8], and we sought to generalize our work to a different viral vaccine model.

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Recent studies have demonstrated that genetic polymorphisms in several genes (MTHFR and IRF1) were associated with adverse events after smallpox vaccination [9]. Data also suggest that certain interleukin (IL)–1, IL-4, and IL-18 gene haplotypes may predict the development of fever after smallpox vaccine [10].

Because HLA alleles are essential for determining the specificity of an individual's immune response, we examined the influence of HLA alleles on variability in immune responses to a licensed smallpox vaccine. Our present study is, to our understanding, the first study to have assessed relationships between HLA gene polymorphisms and both humoral (neutralizing antibody) and cellular (total peripheral blood mononuclear cell [PBMC] of interferon [IFN]–γ by Elispot assays, CD8α^+ IFN-γ levels by Elispot assays, and secreted cytokines) immune responses in healthy individuals to primary smallpox vaccination.

METHODS

Subjects

Our study group included a sample of 1076 healthy, eligible subjects and active-duty personnel who participated in both the US Department of Health and Human Services civilian health care employee smallpox immunization program at the Mayo Clinic (Rochester, MN) and the smallpox immunization program at the US Naval Health Research Center (NHRC; San Diego, CA) [11]. All study subjects received a single dose of a licensed vaccinia (smallpox) vaccine during the period 2002–2006 and had a documented vaccine "take" (ie, development of a pustule at the vaccination site after immunization). The subjects received Dryvax, a lyophilized, live-virus preparation of infectious VACV (Wyeth Laboratories), with a minimum concentration of 10^8 pock-forming units (PFU)/mL [12]. The institutional review boards of both the Mayo Clinic and NHRC approved the study, and we obtained written informed consent before enrollment from each subject.

Neutralizing Antibody Assay

We optimized the VACV-specific neutralization assay using β-galactosidase expressing VACV developed at the US Food and Drug Administration [13, 14]. Outcomes are defined as the serum dilution that inhibits 50% of VACV activity (ID_{50}), which was calculated using the M estimation approach pioneered by Huber [15], which is robust to outliers and is implemented in the Robustreg technique of the SAS software package version 9 (SAS Institute). Each serum sample was tested at least 3 times, with values recorded for each test.

IFN-γ Elispot

Total PBMC IFN-γ ($n = 1058$) and CD8α^+ IFN-γ ($n = 1002$) enzyme-linked immunosorbent spot (ELISPOT) assays (R&D Systems) were performed in cell cultures (rested in the presence of 50 IU/mL IL-2), as described elsewhere [16], after stimulation with inactivated VACV (the New York City Board of Health strain) at a multiplicity of infection (MOI) of 5. The CD8α^+ IFN-γ ELISPOT assay uses detection and visualization of CD8α^+ IFN-γ–secreting cells using plates that are precoated with both human CD8α^+ and human IFN-γ monoclonal antibodies. All plates were analyzed with an ImmunoSpot S4 Pro-Analyzer using ImmunoSpot software, version 4.0 (Cellular Technology).

Cytokine Measurements

PBMCs were plated in 96-well plates at a concentration of 2×10^5 per well. Viral stimulation conditions were optimized [17] and are as follows: for IFN-β ($n = 880$), IL-2 ($n = 871$), and IL-18 ($n = 870$), the MOI was 5 (24 hours); for IL-12p40 ($n = 893$), IL-12p70 ($n = 890$), TNF-α ($n = 921$), and IL-1β ($n = 921$), the MOI was 0.5 (24 hours); for IFN-α ($n = 1038$) and IFN-γ ($n = 1038$), the MOI was 0.05 (4 days); for IL-4 ($n = 1000$) and IL-10 ($n = 1003$), the MOI was 0.05 (7 days); for IL-6 ($n = 847$), the MOI was 5 (8 days). To detect cytokines from culture supernatants, we used enzyme-linked immunosorbent assay (ELISA)–based kits for IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, tumor necrosis factor (TNF)–α, and IFN-γ (BD Pharmingen); IFN-α and IFN-β (PBL Biomedical Laboratories); and IL-18 (MBL International). These cytokines were chosen on the basis of 2 criteria: they are either the targets of poxvirus-encoded proteins or have been shown to play a role in poxvirus infections. The levels of sensitivity for the IL-1β,IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, TNF-α, IFN-γ, IFN-α, IFN-β, and IL-18 assays were 3.9 pg/mL, 7.8 pg/mL, 7.8 pg/mL, 4.7 pg/mL, 7.8 pg/mL, 31.3 pg/mL, 7.8 pg/mL, 7.8 pg/mL, 4.7 pg/mL, 12.5 pg/mL, 250 pg/mL, and 25.6 pg/mL, respectively.

HLA Genotyping

HLA genotyping was performed with high-resolution A-SSP, B-SSP, C-SSP, DRB1-SSP, DQA - SSP, DQB1-SSP, DPA1-SSP, and DPB1-SSP Unitray typing assays with the entire locus on a single tray (Invitrogen), as described elsewhere [6–8]. All polymerase chain reaction amplifications were performed on an ABI-377 (Applied Biosystems) and analyzed using MatchTools (Applied Biosystems) software.

Statistical Methods

The statistical methods described here are similar to those performed for our previous HLA association publications [6–8]. Quantification of antibody titers resulted in ≥ 3 observations per subject. Subject-specific assessments of cytokine secretion and ELISPOT resulted in 3 recorded values before stimulation with VACV and 3 recorded values after stimulation. For descriptive purposes, a single response measurement per individual was obtained by using the median of the ID_{50} values for antibodies

and the difference in the medians of the stimulated and unstimulated values for cytokines and ELISPOT values.

Alleles were grouped on the basis of HLA genotype, and descriptive summaries for the immune measures were obtained using medians and interquartile ranges (IQRs). Individuals contributed 2 observations to these descriptive summaries—one for each allele. HLA associations were then formally evaluated using linear regression. Covariates were included that reflected the ordinal effects of each common allele (those observed $\geq$5 times). Repeated measures analyses were used to simultaneously model the multiple observations per subject. We accounted for within-subject correlations using generalized estimating equations. Differences in immune responses among all alleles of each locus were first assessed globally. For antibody-level analyses, this was achieved by including all but 1 of the ordinal allele variables in a multivariable linear model and by simultaneously testing the significance of the entire set of alleles from that HLA locus. For the analyses of cytokine outcomes, the same set of the allele variables were included in a model for each HLA locus, together with a variable representing stimulation status. Allele-by-stimulation status interactions were then simultaneously tested for statistical significance. After performing these global tests, we examined individual allele associations with immune response. These allelic associations were not considered statistically significant in the absence of locus-specific global significance. All statistical analyses adjusted for sex, age at blood draw (quartiles), time from smallpox immunization to blood draw (quartiles), time from blood draw to assay (quartiles), shipping temperature of the sample, and time of year when the blood sample was shipped (warm weather months [April-September] vs cold weather months [October-March]). Finally, we used single nucleotide polymorphism (SNP) genotype data obtained from a genome-wide association study of the same study subjects to account for the possibility of population stratification. We used autosomal SNP genotypes from the Illumina HumanHap550 to create principal components. The resulting first 3 eigenvectors were included as covariates in all analyses. Data transformations were used to correct for data skewness in all linear regression models. An inverse cumulative normal (probit) transformation was used for the cytokine and ELISPOT variables, and log transformation was used for the antibodies. To address the effects of multiple testing, we supplemented all locus-specific global *P* values with corresponding global Q-values based on the concept of false discovery rate [18]. All statistical tests were 2-sided, and all analyses were performed using the SAS software system version 9 (SAS Institute).

RESULTS

Demographic Characteristics

We studied 1071 healthy individuals aged 18–40 years who had been vaccinated with 1 dose of the United States–licensed smallpox vaccine (Dryvax). One-half of the subjects were white (53%), and the median time from smallpox vaccination to blood draw was 15 months (Table 1). Both humoral and cellular responses to VACV antigens displayed a high degree of variability in the study population. Vaccinia-specific IL-4, IL-10, IL-12p70, IL-18, and IFN-β secretion levels were low and hardly detectable by ELISA. For neutralizing antibody titer analyses and for the following ELISPOT and cytokine secretion analyses, we do not present allele-specific data if the corresponding locus-specific global test result was not statistically significant ($P \leq .05$).

Table 1. Demographic Characteristics of the Study Population

Variable	Number of subjects (percent)
Overall	1,071
Age at enrollment	
18-21 years	242 (23)
22-23 years	259 (24)
24-26 years	303 (28)
27-40 years	267 (25)
Gender	
Female	281 (26)
Male	790 (74)
Race	
African American	186 (17)
Asian, Pacific Islander	74 (7)
Caucasian	570 (53)
More than one race	87 (8)
Other or don't know	154 (14)
Ethnicity	
Hispanic	229 (21)
Non-Hispanic	797 (74)
Don't know	45 (4)
Time since vaccination	
1-9 months	260 (24)
10-14 months	271 (25)
15-33 months	278 (26)
34-49 months	262 (24)

Associations Between HLA Alleles and Humoral Responses

The median ID_{50} for all subjects was 132.59 (IQR, 78.76–206.46). ID_{50} of neutralizing antibody titers between 20 and 32 are thought to be protective [19]. Table 2 (Supplemental Table 1) displays HLA associations with vaccinia-specific neutralizing antibodies. For class I loci and antibody titers, the global *P* values were .375, .044, and .643 for HLA-A, -B, and -C, respectively. Alleles B*1302 (median ID_{50}, 183.73; $P = .036$) and B*3802 (median ID_{50}, 180.85; $P = .011$) appeared to be significantly associated with higher antibody titers, whereas alleles B*4403 (median ID_{50}, 104.00; $P = .004$) and B*4801 (median ID_{50}, 100.99; $P = .027$) were significantly associated with lower neutralizing antibodies. We also found significant associations

Table 2. HLA Allelic Associations With VACV-Specific Humoral Immune Responses

Locus	Allele	Allele counts	Mean	Std Dev	Median response (ID_{50})	Lower quartile (ID_{50})	Upper quartile (ID_{50})	*P* value[a]	Global *P* value (Q-value)[a]
Antibodies (ID_{50})	Overall	2142	162.00	120.60	132.59	78.76	206.46		
HLA-A									.375
HLA-B									**.044 (.22)**
	*1302	35	209.90	146.25	183.73	68.63	292.27	.036	
	*3802	14	219.90	136.66	180.85	119.11	248.59	.011	
	*4403	119	133.30	92.31	104.00	70.93	175.88	.004	
	*4801	16	109.90	60.04	100.99	66.22	141.36	.027	
HLA-C									.643
HLA-DRB1									.168
HLA-DQA1									.182
HLA-DQB1									**.041 (.22)**
	*0302	206	175.30	116.10	151.97	100.06	228.29	.015	
	*0604	70	187.10	126.26	151.38	96.93	247.04	.017	
HLA-DPA1									.304
HLA-DPB1									.931

NOTE. Linear regression analysis. *P* values for antibody titers from linear regression model based on log-transformed values. Repeated measures analyses were used to account for multiple measures of antibody titers per individual. Analyses adjusted for age at blood draw, gender, time from immunization to blood draw, time from blood draw to assay, shipping temperature of the sample, time of year when sample was shipped, assay technician, and the first three eigenvectors from the principal components analysis of genome-wide association study (GWAS) data. Only statistically significant allelic findings ($P \leq .05$) are presented. Statistically significant global *P* values are in bold type. Q-values are also provided for all statistically significant global *P* values.

between HLA-DQB1*0302 and DQB1*0604 alleles and higher antibodies (global P value = .041). Subjects carrying the DQB1*0302 (median ID_{50}, 151.97; $P = .015$) and DQB1*0604 (median ID_{50}, 151.38; $P = .017$) alleles had higher levels of neutralizing antibody titers.

Associations Between HLA Alleles and ELISPOT Responses

The overall median vaccinia-specific total PBMCs IFN-γ spot-forming cells (SFC, per 2×10^5 cells) and $CD8\alpha^+$ IFN-γ SFC (per 2×10^5 cells) were 52 (IQR 24, 88) and 10 (IQR -2, 27), respectively (Table 3, Supplemental Table 2). For class I loci and IFN-γ ELISPOT, the global tests of significance were .729, .398 and .164 for HLA-A, -B, and -C loci, respectively. The global *P* value for the HLA-DRB1 locus and IFN-γ Elispot was marginally significant ($P = .052$). Alleles DRB1*0103 (median SFC 18, $P = .023$), DRB1*1301 (median SFC 43, $P = .044$) and DRB1*1501 (median SFC 50, $P = .016$) had marginally significant associations with lower IFN-γ ELISPOT responses to VACV. DRB1*0301 (median SFC 58, $P = .016$) and DRB1*1001 (median SFC 67, $P = .028$) had potential associations with higher IFN-γ ELISPOT responses.

The global *P* value for the HLA - DQB1 locus and $CD8\alpha^+$ IFN-γ ELISPOT was significant ($P = .027$). Alleles DQB1*0402 (median SFC 7, $P = .008$) and DQB1*0603 (median SFC 4, $P = .002$) had significant associations with lower $CD8\alpha^+$ IFN-γ ELISPOT responses, while DQB1*0201 (median SFC 11, $P = .026$) had significant association with higher $CD8\alpha^+$ IFN-γ ELISPOT response.

Associations Between HLA Alleles and Cytokine Responses

We found no strong associations with class I and class II alleles and IL-2 and IFN-α secretion levels. The associations between HLA alleles and VACV-specific cytokine (IL-1β, IL-6, IL-12p40, IFN-γ, and TNF-α) secretion levels are summarized in Table 4 (Supplemental Table 3). The overall median vaccinia-specific secretion levels (pg/mL) were 50.8 pg/mL (IQR, 25.5–121.9 pg/mL) for IL-1β, 1071.7 pg/mL (IQR, 449.1–1944.5 pg/mL) for IL-6, 62.4 pg/mL (IQR, 29.5–122.4 pg/mL) for IL-12p40, 289.5 pg/mL (IQR, 11.7–1261.2 pg/mL) for IFN-γ, and 165.0 pg/mL (IQR, 91.0–319.2 pg/mL) for TNF-α.

The global tests of significance demonstrated significant associations between IL-1β secretion and the HLA-B locus (global $P = .004$; $Q = .13$). Alleles B*3906 (median, 52.07 pg/mL; $P = .006$) and B*5701 (median, 116.37 pg/mL; $P < .001$) had significant associations with higher IL-1β secretion. In contrast, B*5301 (median, 35.15 pg/mL; $P = .047$) and B*5601 (median, 33.49 pg/mL; $P = .008$) had significant associations with lower IL-1β secretion.

The global *P* value for HLA-B locus and vaccinia-specific IL-6 production was .016 ($Q = .20$). Alleles B*3508 (median, 1445.40 pg/mL; $P = .04$), B*3906 (median, 1939.90 pg/mL; $P = .021$), and B*5701 (median, 1915.40 pg/mL; $P = .035$) demonstrated associations with increased IL-6 secretion. Alleles B*3701 (median, 723.24 pg/mL; $P = .02$), B*4403 (median, 777.43 pg/mL; $P = .033$), and B*5501 (median, 863.42 pg/mL; $P = .033$) were associated with decreased IL-6 levels.

Table 3. HLA Allelic Associations With VACV-Specific Enyme-Linked Immunosorbent Sport (ELISPOT) Immune Responses

Locus	Allele	Allele counts	Mean	Std dev	Median response (SFC 2 × 10^5 cells)	Lower quartile (SFC 2 × 10^5 cells)	Upper quartile (SFC 2 × 10^5 cells)	*P* value[a]	Global *P* value (Q-value)[a]
IFN-γ Elispot (SFC 2 × 10^5 cells)	Overall	2116	61	57	52	24	88		
HLA-A									.729
HLA-B									.398
HLA-C									.164
HLA-DRB1									.052 (.22)
	*0103	21	25	40	18	6	53	.023	
	*0301	213	64	57	58	25	91	.016	
	*1001	26	72	51	67	30	114	.028	
	*1301	91	58	58	43	17	83	.044	
	*1501	192	57	51	50	25	83	.016	
HLA-DQA1									.329
HLA-DQB1									.200
HLA-DPA1									.719
HLA-DPB1									.387
$CD8\alpha^+$ IFN-γ Elispot (SFC 2 × 10^5 cells)	Overall	2004	15	40	10	−2	27		
HLA-A									.593
HLA-B									.522
HLA-C									.229
HLA-DRB1									.114
HLA-DQA1									.079
HLA-DQB1									**.027 (.20)**
	*0201	202	18	37	11	0	28	.026	
	*0402	117	19	62	7	−4	27	.008	
	*0603	90	7	21	4	−4	16	.002	
HLA-DPA1									.266
HLA-DPB1									.255

NOTE. [a]Linear regression analysis. *P* values for cytokine levels were based on inverse cumulative normal transformation. Repeated measures analyses were used to account for multiple measures of cell counts per individual. Analyses adjusted for age at blood draw, gender, time from immunization to blood draw, time from blood draw to assay, shipping temperature of the sample, time of year when sample was shipped, assay technician, and the first three eigenvectors from the principal components analysis of genome-wide association study (GWAS) data. Only statistically significant allelic findings ($P \leq .05$) are presented. SFC - spot-forming cells per 2 × 10^5 cells. Statistically significant global *P* values are in bold type. Q-values are also provided for all statistically significant global *P* values.

For the class I A locus and IL-12p40 production, the global *P* value was significant ($P = .042$; $Q = .22$). Alleles A*1101 (median, 51.42 pg/mL; $P = .023$) and A*3402 (median, 35.51 pg/mL; $P = .028$) demonstrated significant associations with decreased IL-12p40 secretion, whereas A*3101 (median, 94.97 pg/mL; $P < .001$) was significantly associated with increased IL-12p40 secretion.

Regarding vaccinia-induced IFN-γ secretion, no association of the class I HLA loci was globally significant, nor was any association with the class II DPA1 and DPB1 loci (Table 4). For other class II loci and IFN-γ production, the global *P* values (Q-values) were .019 (Q = .20) for HLA-DRB1, .003 (Q = .13) for HLA-DQA1, and .055 (Q = .22) HLA-DQB1. Alleles DRB1*0101 (median, 151.01 pg/mL; $P = .002$), DRB1* 0401 (median, 133.16 pg/mL; $P = .028$), DRB1* 1101 (median, 178.42 pg/mL; $P = .010$), and DRB1*1103 (median, 259.73 pg/mL; $P = .022$) were significantly associated with lower IFN-γ production. Alleles DRB1*0301 (median, 360.74 pg/mL; $P = .027$) and DRB1*0701 (median, 521.32 pg/mL; $P = .016$) were associated with increased IFN-γ secretion. For the HLA-DQA1 locus, alleles DQA1*0201 (median, 508.37 pg/mL; $P = .019$) and DQA1*0501 (median, 353.92 pg/mL; $P = .032$) were associated with increased IFN-γ secretion, whereas DQA1*0101 (median ,184.44 pg/mL; $P = .007$) was significantly associated with lower IFN-γ secretion. For the HLA-DQB1 locus and IFN-γ secretion, the global *P* value for association did not reveal a convincing statistically significant association ($P = .055$). The outcomes of our following analyses by allele for the DQB1 locus implied a possible association with DQB1*0201 (median, 415.94 pg/mL; $P = .041$) and DQB1*0501 (median, 193.17 pg/mL; $P = .004$)

Table 4. HLA Allelic Associations With VACV-Specific Cytokine Immune Responses

Cytokine/Locus	Allele	Allele counts	Mean (pg/mL)	Std dev	Median response (pg/mL)	Lower quartile (pg/mL)	Upper quartile (pg/mL)	*P* value[a]	Global *P* value (Q-value)[a]
IL-1β (pg/mL)	Overall	1842	105.40	178.22	50.80	25.51	121.87		
HLA-A									.480
HLA-B									**.004 (.13)**
	*3906	18	128.10	219.74	52.07	26.63	93.33	.006	
	*5301	48	64.33	91.02	35.15	19.66	74.78	.047	
	*5601	12	45.96	53.21	33.49	20.19	61.94	.008	
	*5701	43	171.40	154.59	116.37	53.55	260.88	<.001	
HLA-C									.190
HLA-DRB1									.622
HLA-DQA1									.349
HLA-DQB1									.638
HLA-DPA1									.783
HLA-DPB1									.226
IL-6 (pg/mL)	Overall	1694	1275.07	1140.13	1071.67	449.09	1944.52		
HLA-A									.202
HLA-B									**.016 (.20)**
	*3508	14	2039.00	1846.70	1445.40	730.77	3844.80	.040	
	*3701	19	790.00	1413.20	723.24	47.71	1883.80	.020	
	*3906	16	1889.00	1206.50	1939.90	887.62	2486.40	.021	
	*4403	92	1057.00	1195.40	777.43	230.80	1690.30	.033	
	*5501	21	984.70	1066.60	863.42	114.50	1400.50	.033	
	*5701	40	1872.00	1277.00	1915.40	702.83	2592.20	.035	
HLA-C									.889
HLA-DRB1									.222
HLA-DQA1									.284
HLA-DQB1									.852
HLA-DPA1									.323
HLA-DPB1									.083
IL-12p40 (pg/mL)	Overall	1786	92.62	120.81	62.41	29.48	122.38		
HLA-A									**.042 (.22)**
	*1101	90	62.82	119.30	51.42	22.96	91.85	.023	
	*3101	52	142.70	235.22	94.97	48.30	152.28	<.001	
	*3402	14	51.67	55.63	35.51	8.12	108.05	.028	
HLA-B									.351
HLA-C									.699
HLA-DRB1									.193
HLA-DQA1									.613
HLA-DQB1									.388
HLA-DPA1									.606
HLA-DPB1									.460
IFN-γ (pg/mL)	Overall	2076	864.09	1648.61	289.51	11.71	1261.24		
HLA-A									.442
HLA-B									.245
HLA-C									.314
HLA-DRB1									**.019 (.20)**
	*0101	107	626.80	1383.20	151.01	−60.40	934.36	.002	
	*0301	209	1070.00	1879.20	360.74	31.30	1469.20	.027	
	*0401	108	831.30	1586.60	133.16	3.57	1279.90	.028	

Table 4. (*Continued*)

Cytokine/Locus	Allele	Allele counts	Mean (pg/mL)	Std dev	Median response (pg/mL)	Lower quartile (pg/mL)	Upper quartile (pg/mL)	*P* value[a]	Global *P* value (Q-value)[a]
	*0701	236	1115.00	1586.10	521.32	84.53	1590.30	.016	
	*1101	111	590.90	1394.10	178.42	−13.81	718.18	.010	
	*1103	11	1078.00	1974.50	259.73	31.70	1269.00	.022	
HLA-DQA1									**.003 (.13)**
	*0101	170	636.00	1447.90	184.44	−42.55	934.36	.007	
	*0201	240	1095.00	1568.10	508.37	82.98	1534.00	.019	
	*0501	221	1057.00	1847.90	353.92	31.30	1469.20	.032	
HLA-DQB1									**.055 (.22)**
	*0201	209	1086.00	1882.40	415.94	31.30	1479.20	.041	
	*0501	231	690.40	1545.10	193.17	−42.15	934.36	.004	
HLA-DPA1									.362
HLA-DPB1									.113
TNF-α (pg/mL)	Overall	1842	218.42	228.61	165.02	91.05	319.25		
HLA-A									.315
HLA-B									**.006 (.13)**
	*3502	21	139.50	122.58	106.47	81.34	151.75	.009	
	*5601	12	106.20	73.08	89.02	58.67	148.14	.004	
	*5701	43	349.90	220.08	320.90	169.00	477.71	<.001	
HLA-C									.566
HLA-DRB1									.151
HLA-DQA1									.129
HLA-DQB1									.395
HLA-DPA1									.880
HLA-DPB1									.071

NOTE. [a]Linear regression analysis. *P* values for cytokine levels were based on inverse cumulative normal transformation. Repeated measures analyses were used to account for multiple measures of cytokine secretion per individual. Analyses adjusted for age at blood draw, gender, time from immunization to blood draw, time from blood draw to assay, shipping temperature of the sample, time of year when sample was shipped, and the first three eigenvectors from the principal components analysis of genome-wide association study (GWAS) data. Only statistically significant allelic findings ($P \leq .05$) are presented. Statistically significant global *P* values are in bold type. Q-values are also provided for all statistically significant global *P* value.

and variation in IFN-γ secretion. These suggestive associations should be taken with caution because of the lack of a significant global test.

Finally, our analysis by allele for the HLA-B locus found associations between lower TNF-α secretion and B*3502 (median, 106.47 pg/mL; $P = .009$) and B*5601 (median, 89.02 pg/mL; $P = .004$). In addition, B*5701 (median, 320.90 pg/mL; $P < .001$) had a significant association with higher TNF-α secretion.

DISCUSSION

Smallpox vaccination leads to strong T and B lymphocyte immune responses [1, 20, 21]. Our results suggest that certain class I B (*1302, *3802, *4403, and *4801) and class II DQB1 (*0302 and *0604) alleles are significantly associated with VACV-specific divergent antibody response levels. The association of the B*4403 and B*4801 alleles with decreased antibody production has significance with respect to immune estimates of protection against smallpox and with respect to future epitope HLA-based smallpox vaccine design.

With regard to smallpox vaccine-induced cellular immunity, at least 3 relatively common HLA - DQB1 alleles were associated with significantly increased (*0201) and decreased (*0402 and *0603) $CD8\alpha^+$ IFN-γ ELISPOT responses. This suggests that class II polymorphisms are able to direct and/or influence the antigen (vaccinia)–specific T-cell ELISPOT response. Given that cytokines are intracellularly produced molecules that control the strength of the immune response by modulating cell activation and differentiation [22], the associations between HLA alleles and vaccinia-specific cytokine secretion were also examined.

Specific HLA class I alleles were previously found to be associated with cytokine immune responses to viral vaccines [23–25]. In a study of cytotoxic T lymphocyte (CTL) responses of PBMCs from humans vaccinated with Dryvax, Oseroff et al [26] found that ~35 different VACV proteins are recognized by class I HLA-restricted responses. In our study, several HLA-A alleles (*1101, *3101, and *3402) were associated with variations

in IL-12p40 secretion. We also observed the strongest class I associations between multiple alleles of the HLA-B locus and variations in IL-1β (*3906, *5301, *5601, and *5701), IL-6 (*3508, *3701, *3906, *4403, *5501, and *5701) and TNF-α (*3502, *5601, and *5701) secretion. Thus, a broad class I-restricted T-cell response appears to be particularly important in smallpox vaccine-induced immunity.

Importantly, the B*5701 allele, which appears with a frequency of 2%-7% in human populations (http://www.allelefrequencies.net/) [27], was associated with increased levels of IL-1β, IL-6, and TNF-α secretion. With regard to infectious diseases and vaccines, the B*5701 allele has been consistently associated with slower disease progression to human immunodeficiency virus (HIV) infection and control of virus reproduction in HIV-infected individuals [28–31]. A study of recombinant canarypox vaccine (ALVAC-HIV-1) suggested that the B*57 allele was associated with an earlier and more robust CTL response [31]. The protective and enhancing effect of the B*5701 allele against HIV and increased cytokine responses after smallpox vaccine could be a novel finding in smallpox vaccine-induced immunity.

It is important to mention that specific class II alleles have also been implicated in infectious diseases and in immune responses to vaccines, including measles, rubella, mumps, and hepatitis B [7, 32–34]. It was previously reported that 2 alleles, DRB1*0301 and DQB1*0201, were significantly associated with lower measles vaccine-induced IFN-γ secretion [35]. In addition, DRB1*0101 and DQB1*0501 alleles were also demonstrated to be associated with higher rubella vaccine-induced IFN-γ secretion [25]. Similarly, the impact of class II polymorphisms on smallpox vaccine-induced IFN-γ responses is striking. It is reasonable to presume that some class II molecules present naturally processed VACV epitopes more efficiently to IFN-γ-secreting $CD4^+$ (or natural killer) cells, resulting in a strong antiviral immune response. In our study, several common alleles of the DRB1 (*0101, *0401, *0701, and *1101), DQA1 (*0101 and *0201), and DQB1 (*0501) loci were associated with lower IFN-γ secretion levels. In contrast, several alleles of the DRB1 (*0301 and *1103), DQA1 (*0501), and DQB1 (*0201) loci were associated with increased IFN-γ secretion. Some of these alleles are consistent with previous observations that specific class II alleles have been reported to confer a protective effect from infections of HIV, hepatitis C virus, and hepatitis B virus, and some of these were consistently associated with measures of immune responses to vaccinations [36–38]. For example, studies have demonstrated an association between the DRB1*07 allele and its haplotype, DRB1*07-DQB1*02, as well as low antibody response to hepatitis B vaccine [39–41].

This study represents the first study to investigate the role of HLA gene polymorphisms and immune responses to a licensed smallpox vaccine. The strengths of our study design include a well-characterized study cohort with documented single-dose Dryvax vaccine coverage, availability of good demographic data from vaccinees, and the relatively large sample size. The quantitative smallpox vaccine–specific humoral and cellular immune profiling of our study subjects permitted us to look for HLA allele variations in addition to cross-regulation patterns for HLA variants, which increased our confidence in the genotype-phenotype associations we found. There are also limitations to the present study. The issues of multiple tests and potential false-positive associations are of concern in studies such as this. Many statistical tests were performed for this study; thus, the possibility of false-positive results is present. We reduced the chance of a type-I error by examining allelic effects only in the presence of a significant global locus effect. Nevertheless, this still resulted in a sizable number of global tests: our analyses examined 15 immune response outcomes, each on 8 HLA loci, resulting in a total of 120 global tests. Assuming sovereign tests of association, one would expect about 6 of these tests to be statistically significant at the $P = .05$ level. We found a total of 9 significant tests at the .05 level and another 2 significant at just above the .05 level ($P = .052$ and .055)—comfortably more than estimated. Several of the Q values were below the .15 level, suggesting that those associations have a >85% chance of being true-positive results. All P values of ≤.055 were estimated to have a >75% chance of being true-positive results. However, because this is the first study of its kind to examine immune responses to smallpox vaccination, there is a need to validate these results with independent data from replication studies [42]. Because immune responses to vaccination occur in the context of the complex interaction of host genetic traits, the role of HLA supertypes and haplotypes in variable responses to smallpox vaccine should be also examined in future studies. We believe that it is also important to assess the role of other immune response genes, acting alone or in concert with other genetic determinants, and class III genes in extended HLA class I–class II haplotypes or regions in linkage disequilibrium in smallpox vaccine-induced immunity, consistent with our immune-response network theory [43–45].

In conclusion, we demonstrated that specific HLA class I and II alleles are associated with significant variations in both humoral and cell-mediated immune responses to VACV. We found significant associations between specific alleles of the class I B locus and variations in vaccinia neutralizing antibody levels and IL-1β, IL-6, and TNF-α cytokine immune responses. Distinctively, the B*5701 allele was associated with increased levels of vaccinia-induced IL-1β, IL-6, and TNF-α secretion levels and could be considered an important allele–influencing vaccinia-specific cell-mediated immune response. We also demonstrated that class II DRB1, DQA1, and DQB1 gene polymorphisms influence vaccinia-induced IFN-γ responses. It is also possible that these HLA alleles are linked to other genes associated with immune responses to VACV. Additional studies are required to confirm these results and to reproduce these

HLA class I and II associations (ie, HLA profiles) in different populations. Because HLA genes are important components of the immune system, characterization of HLA profiles could help to identify immunodominant vaccinia epitopes that could be integrated in future improved smallpox vaccine development approaches [27].

It is estimated that 30%–50% of the US population has contraindications to receiving current live smallpox vaccines, and given the importance of developing better smallpox vaccines for widespread population use, data from this study could inform directed development of next generation smallpox vaccines [43–45]. Such information may also provide general principles that may guide the rational development of other new vaccines against viral agents of bioterrorism by determining the basis for genetic disparity in vaccine-induced immune responses [46].

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