Host genetic determinants of T cell responses to the MRKAd5 HIV-1 gag/pol/nef vaccine in the Step trial

Running title: Host genetics of T cell responses in Step

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Abstract

Understanding how human genetic variation impacts individual response to immunogens is fundamental for rational vaccine development. To explore host mechanisms involved in cellular immune responses to the MRKAd5 HIV-1 gag/pol/nef vaccine tested in the Step trial, we performed a genome-wide association study of determinants of HIV-specific T cell responses, measured by IFN-γ ELISpot assays. No human genetic variant reached genome-wide significance, but polymorphisms located in the Major Histocompatibility Complex region showed the strongest association with response to the HIV-1 Gag protein: HLA-B alleles known to associate with differences in HIV-1 control were responsible for these associations. The implication of the same HLA alleles in vaccine-induced cellular immunity and in natural immune control is of relevance for vaccine design. Furthermore, our results demonstrate the importance of considering the host immunogenetic background in the analysis of immune responses to T cell vaccines.

Key words:

HIV vaccine

Genome-wide association study

Cellular immunity

T cell immunity

Host genetics

Immunogenetics

The Step trial was a Phase IIb proof-of-concept study conducted by the HVTN and Merck designed to assess the safety and efficacy of a 3-dose regimen of the Merck adenovirus serotype 5 HIV-1 gag/pol/nef vaccine (MRKAd5 HIV-1 gag/pol/nef), a replication-defective Ad5 vaccine expressing Gag, Pol, and Nef proteins from an HIV-1 Clade B strain, aiming to elicit cellular immune responses. It was a multicenter, double-blind, randomized, placebo controlled study performed in adults at high risk of HIV-1 infection [1]. Vaccinations in the trial were terminated following a planned interim analysis in September 2007 because of a lack of efficacy of the tested vaccine. Vaccination was not associated with a lower viral load in subjects who became infected, and the rate of HIV-1 acquisition was actually higher among vaccine recipients than in placebo recipients, particularly in the subgroup of uncircumcised male subjects with high pre-existing adenovirus serotype 5 (Ad5) neutralizing antibody titers [1, 2]. Understanding this disappointing and paradoxical outcome is a high priority for the field.

Recombinant Ad5 vaccine candidates had previously been shown to elicit strong cellular immune responses against HIV-1 epitopes [3, 4]. Accordingly, the MRKAd5 HIV-1 gag/pol/nef vaccine was highly immunogenic for inducing HIV-specific T cell responses: at least three-quarters of vaccine recipients generated a response to one or more of the HIV-1 proteins included in the vaccine [2]. Still, those responses were quantitatively highly variable: higher response rates were observed in participants with low pre-existing Ad5 neutralizing antibody titers [2], but a significant fraction of variability in response intensity remains unexplained and could be at least partially attributable to human genetic variation.

Thanks to recent advances in genomics, technology and bioinformatics, it is now possible to comprehensively assess associations of common human genetic variants with biological phenotypes [5]: here we combine whole-genome genotyping results and HLA class I typing data to explore the host

mechanisms associated with variability in T cell responses to the HIV-1 proteins present in the MRKAd5 HIV-1 gag/pol/nef vaccine tested in the Step trial.

Methods

Subjects: Study participant enrollment inclusion and exclusion criteria for the Step study have been described previously [1]. Each participant provided informed consent for genetic testing. All male Step trial participants who received at least two doses of the MRKAd5 HIV-1 gag/pol/nef vaccine and were HIV-1 seronegative at week eight, i.e., four weeks after the second vaccination, were eligible for our study. This investigation was initiated after the interim analysis when all but one of the cases identified were males [1]. Most immunogenicity and covariate analyses subsequently performed were confined to male participants, and accordingly this study was restricted to male vaccine recipients.

Immunological assays: Validated interferon gamma (IFN-γ) ELISpot assays were run on previously cryopreserved PBMCs that were obtained at week eight: the Merck laboratory (Merck) tested a quarter of the samples, randomly selected and stratified by treatment assignment and study site. The HVTN laboratory at Fred Hutchinson Cancer Research Center laboratory (HVTN) tested the remaining samples. Cells were stimulated *ex vivo* with pools of peptides that were 15 amino acids in length and overlapping in sequence by 11 amino acids. The peptide sequences matched the HIV-1 proteins encoded by the vaccine, and a total of four non-overlapping pools of peptides were tested: one Gag, two Pol, and one Nef pools. If any of the protein-level responses was positive, the overall response was considered positive. Responses were reported as the number of spot-forming cells per million PBMC. A sample was considered positive if the background-adjusted mean of the experimental wells was ≥ 4 x the mean of the negative controls and ≥55 SFC/10^6 PBMC for the assays run at the Merck Laboratory, while positivity

was assessed using a bootstrapping method specifically developed for in-house ELISpot data for the samples run at HVTN [6, 7]. We also compared the HIV-1 specific IFN-γ ELISpot results to CD4+ and CD8+ T-cell responses measured on a subset of samples by IFN-γ and interleukin-2 (IL-2) intracellular cytokine staining (ICS) assays [2].

Genotyping: DNA samples were genotyped using the Human1M-Duo Infinium HD BeadChip (Illumina), which features more than one million single nucleotide polymorphisms (SNPs) and an additional 52,167 markers designed to specifically target copy number variant regions. We also inferred larger copy number variants from the genotyping data using the PennCNV software [8]. A series of data cleaning and quality control procedures were carried out: SNPs were filtered based on missingness (dropped if called in <99% of subjects), minor allele frequency (dropped if <0.005) and Hardy-Weinberg Equilibrium deviation. Study subjects were filtered based on genotyping quality, a gender check (heterozygosity testing) and cryptic relatedness (sharing of genetic information was first predicted by estimating identity by descent (IBD); then one sample in each pair of DNA samples showing >12.5% of IBD was excluded). High-resolution HLA class I typing (4 digits HLA-A, HLA-B and HLA-C) was obtained using sequence-based methods.

Association analysis: SNPs, copy number variants and HLA class I alleles were tested for association with IFN-γ ELISpot responses to Gag, Pol and Nef in regression models that also included age, pre-vaccination Ad5 serology titers and the laboratory in which the ELISpot assay was performed (HVTN vs. Merck) as covariates. In addition, to correct for population stratification, we applied a modified EIGENSTRAT method [9], which is a principal component analysis of the genotyping data, to define ethnic groups and correct for residual population ancestry within each group. The analyses were run separately in three ethnic groups using PLINK [10]. Combined P values for all three populations were

obtained using the Stouffer's weight Z-method [11]. We used linear regression to test for association between genetic variants and natural log-transformed quantitative ELISpot responses, and logistic regression for qualitative assessment of the ELISpot responses (case-control comparisons between individuals with or without detectable responses to the HIV-1 peptide pools). Significance was assessed with a straight Bonferroni correction (threshold for genome-wide significance: P=5E-08). Power calculations were performed using the PowerCalc software (Lan L, Wang S & Feng S. GWASPower/QT: a statistical power calculation software for genome-wide association studies with quantitative traits in natural populations, submitted), available at http://www.genome.duke.edu/labs/goldstein/software.

Results

Participants and samples: A total of 831 subjects were eligible for the study. They all were male vaccine recipients and HIV-1 negative at week eight. However, 36 of them became infected later in the study. Participants were enrolled in nine countries in the Americas and Australia, and self-reported ethnicities were very diverse, with a majority of white and multi-racial individuals (Table 1). All DNA samples were run on Human1M-Duo chips: 22 samples were excluded because of insufficient genotyping quality; three individuals were excluded because of cryptic relatedness: one in each of two pairs of 100% identical genotypes and one in a pair of 50% identical genotypes; and two subjects were excluded because of a gender discrepancy between genotype and the phenotypic database (females misclassified as males).

Immunological assays: Of the 831 samples, 792 had sufficient cryopreserved PBMCs to be tested in IFN-γ ELISpot assays: 30.6% were analyzed by Merck (N=242) and 69.4% by the HVTN (N=550). IFN-γ ELISpot responses were detected in 85.2% (675/792) of the vaccine recipients. The percentages of subjects with positive responses were 75.6% for Gag, 69.3% for Nef, and 63.6% and 55.3% for the two

Pol peptide pools. Gag-specific expression of IFN-γ and/or IL-2 measured by ICS assays gated on CD4+ and CD8+ T-cell populations, available for 150 samples [2], were compared to Gag-specific responses measured by IFN-γ ELISpot assays (Figure 1): we observed a strong correlation between ELISpot results and CD8+ T-cell responses (r2=0.4, P<0.001), but no correlation with CD4+ T-cell responses (r2=0.05, P=0.1).

Population structure: Altogether, 768 subjects had complete genotype and phenotype results (Table 1). To avoid spurious associations due to population structure, we ran separate analyses in three ethnic groups corresponding to the major clusters identified in the principal component analysis of the genotyping data. After removal of individuals who could not be classified into a homogeneous group, a total of 318 white, 87 black and 115 Mestizo subjects were available for regression analyses (Table 2). The study had 80% power to detect genetic variants explaining at least 6.6% of the variability in quantitative HIV-1 specific responses in the combined population.

Association analysis: The genome-wide analyses did not reveal any significant association between IFN-γ ELISpot responses to Gag, Pol or Nef and SNPs or copy number variants after correction for multiple testing. However, in the analysis of association with quantitative responses to Gag, the SNPs with the lowest P values clustered in the Major Histocompatibility Complex (MHC) region, around the *HLA-B* and *HLA-C* genes (Figure 2); no such clustering was observed for Pol or Nef associations. Therefore, we tested all 4-digit HLA class I alleles present in the study population for association with the same phenotype. In whites with positive Gag-specific responses (N=265), three HLA-B alleles associated with higher response: HLA-B*2705 (N=27, P=4.6E-05), B*5101 (N=29, P=0.02) and B*5701 (N=17, P=0.05); whereas two alleles associated with lower responses: HLA-B*0801 (N=60, P=2.7E-04) and B*4501 (N=5, P=0.01) (Figure 3). Together, these HLA-B alleles accounted for 13.6% of the variability

in Gag responses. No significant association was found in the smaller groups of black and Mestizo individuals, although a trend toward higher Gag responses was observed for HLA-B*5703 (P=0.1). We did not observe any significant association between HLA-C alleles and quantitative responses to Gag.

We then tested whether the identified HLA-B alleles were responsible for the association signals detected in the MHC region in the genome-wide scan, using nested linear regression. When added to regression models that already incorporated HLA-B*2705, B*5101, B*5701, B*0801 and B*4501 as covariates, the SNPs that showed the strongest associations in the GWAS did not result in a significant increase in the explained variation, confirming that the GWAS signals are indeed explained by the combined effect of functional HLA alleles (Table 3).

Discussion

In an attempt to better delineate the host mechanisms involved in the immune response to T cell vaccines, we performed a genome-wide association study searching for human genetic determinants of the inter-individual variability observed in cellular immune responses to the MRKAd5 HIV-1 gag/pol/nef vaccine. HIV-1 specific T cell responses were measured by IFN-γ ELISpot assays in a large cohort of vaccine recipients. We observed a good correlation between the magnitudes of T cell responses detected by IFN-γ ELISpot and by CD8+ intracellular cytokine staining, which is not surprising since the vaccine preferentially induced CD8+ T cells, and the CD4+ T cells elicited primarily secreted IL-2 and TNF-α [2]. Although no SNP reached genome-wide significance, polymorphisms located in the MHC region showed the strongest association with responses to Gag, of all tested polymorphisms, leading us to look for possible associations between HLA class I alleles and that immunological outcome.

Several HLA-B alleles were found to be responsible for the SNP-associated signal: HLA-B*2705, B*5101 and B*5701 associated with higher Gag responses, whereas HLA-B*0801 and B*4501 associated with lower responses. Strikingly, all five alleles are also known to associate with differences in HIV-1 control [12]. HLA-B*57 is the human genetic factor that has been most consistently associated with potent control of HIV-1 [13-17], with B*5701 observed almost exclusively in Caucasians and B*5703 mostly found in individuals of African ancestry. There is clear epidemiological and functional evidence for effective restriction of HIV-1 by HLA-B*27 [18, 19]. HLA-B*5101 was also known to associate with slower progression to AIDS [20], and its protective effect was nicely confirmed in a recent study that showed that the allele drives HIV-1 adaptation at a population level [21]. Conversely, HLA-B*0801 and HLA-B*4501 have both been associated with higher viral load and more rapid disease progression [20, 22, 23]. The identification of the same HLA alleles, acting in comparable directions,

strongly suggests that overlapping mechanisms are implicated in the development of vaccine-induced cellular immunity to HIV-1 and of natural immune control of HIV-1. Of course, the ELISpot results used in this study only provide information about the immunogenicity of the vaccine, and do not represent a correlate of protection for the tested vaccine. It is possible that the vaccine elicited appropriate CD8+ T cell immune responses to enhance viral control, as suggested by recent data [24], but that these are either too weak in function or magnitude, in too small a subset of the population, or both to confirm meaningfully enhanced control at a population level.

Comparable results had been reported previously in an immunogenetic study of an HIV-1 vaccine. Responses to HIV-1 proteins were measured in samples from ALVAC-HIV recombinant canarypox vaccine trials using a lytic cytotoxic T-lymphocyte assay, and the proportions of samples responding to Gag or Env were found to be significantly higher among those carrying HLA-B*27 or B*57 [25]. The canarypox T cell vaccine that was used was poorly immunogenic for eliciting CD8+ T cells and the study population was smaller, still the concordance between our observations and those earlier results strengthens the case for an important role of protective HLA-B alleles in differential cellular immune responses to HIV-1 vaccines.

Gag-specific CD8+ T-cell responses have repeatedly been related to improved immune control of HIV-1 infection [12, 26, 27], explained largely because Gag is both highly immunogenic and highly conserved in sequence, and because mutational escapes often result in a decrease in viral fitness. The results presented here show that Gag-specific immune responses to T-cell vaccines are modulated by the host immunogenetic background. HLA class I typing data should thus be discussed at the design stage and included in the analysis of future studies of candidate vaccines eliciting robust CD8+ T cells.

Finally, it is noteworthy that most of the inter-individual variation in cellular responses to the MRKAd5 HIV-1 gag/pol/nef vaccine remains unexplained. To help understand the rest of the variation, it seems appropriate to perform additional studies, for example applying resequencing technology to uncover rare causal genetic variants, since we here confirmed the existence of a connection between natural viral control and vaccine response.

Footnotes

Conflicts of interest

DRC and MNR are employees and shareholders of Merck. MJM has served as investigator on Merck-funded research. JF, NF, KVS, ETC, BFH, DEG and DBG declare that they have no conflict of interest.

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Table 1. Characteristics of study participants

		Eligible	In association analyses
Male gender, N (%)		831 (100)	768 (100)
Vaccine recipient, N (%)		831 (100)	768 (100)
Age, mean (SD)		30.4 (7.7)	30.4 (7.7)
Circumcision, N (%)*		458 (55.8)	429 (55.9)
HIV infection during trial, N (%)		36 (4.3)	34 (4.4)
Ethnicity, N (%)	***White***	410 (49.3)	375 (48.8)
	Black	84 (10.1)	76 (9.9)
	Hispanic American	72 (8.7)	69 (9.0)
	Asian	13 (1.6)	13 (1.7)
	Native American	8 (1.0)	7 (0.9)
	Polynesian	1 (0.1)	1 (0.1)
	Multi-racial	242 (29.1)	226 (29.4)
Country, N (%)	***United States***	484 (58.2)	449 (58.5)
	Peru	217 (26.1)	202 (26.3)
	Brazil	50 (6.0)	47 (6.1)
	Canada	27 (3.3)	24 (3.1)
	Haiti	18 (2.2)	15 (2.0)
	Dominican Republic	13 (1.6)	11 (1.4)
	Australia	10 (1.2)	9 (1.2)
	Puerto Rico	10 (1.2)	10 (1.3)
	Jamaica	2 (0.2)	1 (0.1)

* Information missing for 10 subjects

Table 2. Participants included in linear regression analyses, by ethnic group.

Ethnic group	Total N	Positive responses to any HIV-1 peptide pool		Positive Gag-specific responses	
		N	***%***	***N***	***%***
Whites	318	283	89.0	265	83.3
Blacks	87	72	87.8	59	73.0
Mestizos *	115	101	82.8	84	67.8

* Mestizo ("mixed race") is used by Peruvians to describe their ethnic group.

Table 3. The HLA-B alleles HLA-B*2705, *5101, *5701, *0801 and *4501 together explain most of the genome-wide association signals detected in the region. Results are shown for the 5 most associated SNPs in the analysis of Gag-specific responses in whites. All linear regression models include age, Ad5 titers, laboratory (HVTN vs. Merck) and EIGENSTRAT values as covariates. The adjusted P value (‡) is obtained for each SNP in a model in which the 5 HLA-B alleles are incorporated.

Top 5 SNPs in GWAS	**P values for SNP associations**	
	GWAS P value	***Adjusted P value ‡***
rs4713462	1.9E-06	0.02
rs4713460	2.4E-06	0.03
rs2247056	1.4E-05	0.07
rs2853935	1.4E-05	0.06
rs2523535	2.2E-05	0.11

Figure legends

Figure 1. Comparison between Gag-specific ELISpot and ICS results: IFN-γ ELISpot and CD8+ T cell IFN-γ/IL-2 ICS results strongly correlate (upper panel), whereas there is no correlation between IFN-γ ELISpot and CD4+ T cell IFN-γ/IL-2 ICS results (lower panel).

Figure 2. Genomic overview of the MHC region that includes the most associated variants. Indicated are the P values [-log10(P)] for association with Gag-specific responses of all genotyped SNPs in the region and the structures of the surrounding genes. A P value <5E-08 was required to declare significance at the genome-wide level.

Figure 3. HLA-B alleles associated with quantitative T cell responses to Gag in the IFN-γ ELISpot assay. Only white participants are included.

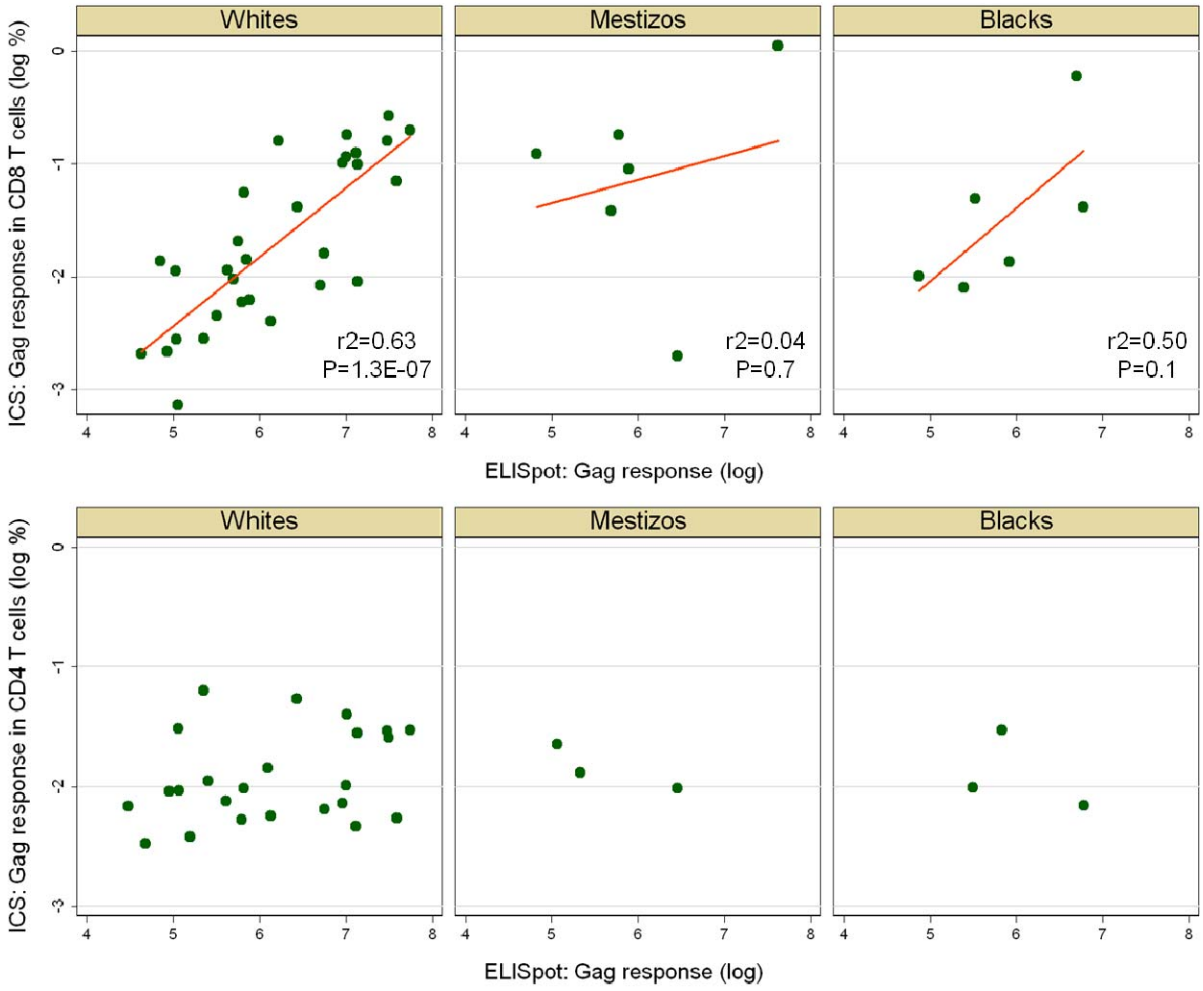
Whites
Mestizos
Blacks
ICS: Gag response in CD8 T cells (log %)
r2=0.63
P=1.3E-07
r2=0.04
P=0.7
r2=0.50
P=0.1
ELISpot: Gag response (log)
Whites
Mestizos
Blacks
ICS: Gag response in CD4 T cells (log %)
ELISpot: Gag response (log)

Figure 1

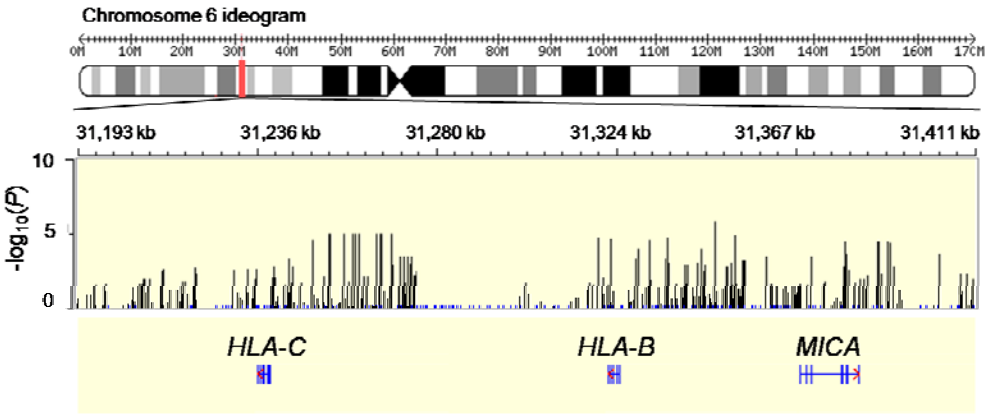
Chromosome 6 ideogram
0M
10M
20M
30M
40M
50M
60M
70M
80M
90M
100M
110M
120M
130M
140M
150M
160M
170M
31,193 kb
31,236 kb
31,280 kb
31,324 kb
31,367 kb
31,411 kb
10
5
0
-log10(P)
HLA-C
HLA-B
MICA

Figure 2

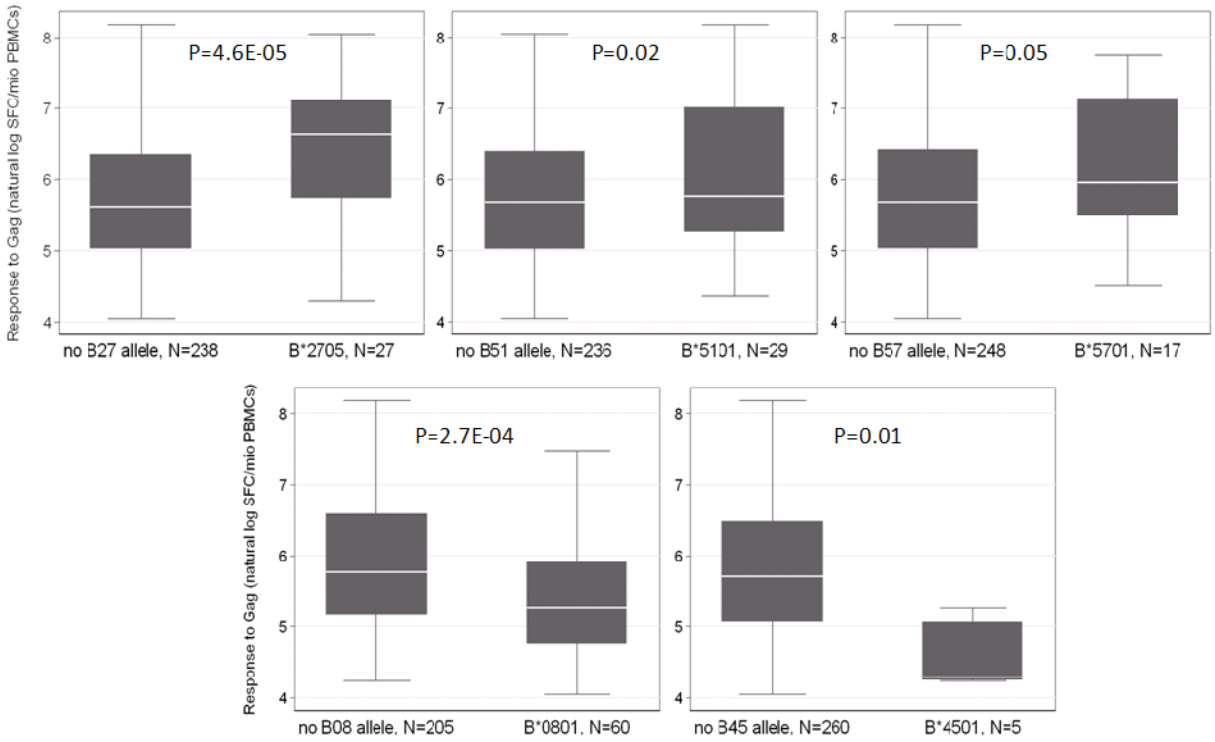
Response to Gag (natural log SFC/mio PBMCs)
P=4.6E-05
no B27 allele, N=238
B*2705, N=27
P=0.02
no B51 allele, N=236
B*5101, N=29
P=0.05
no B57 allele, N=248
B*5701, N=17
P=2.7E-04
no B08 allele, N=205
B*0801, N=60
P=0.01
no B45 allele, N=260
B*4501, N=5

Figure 3