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Genome-Wide Genetic Associations with IFNγ Response to Smallpox Vaccine

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Abstract

Smallpox is a deadly and debilitating disease that killed hundreds of millions of people in the past century alone. The use of vaccinia-virus based smallpox vaccines led to the eradication of smallpox. These vaccines are remarkably effective, inducing the characteristic pustule or “take” at the vaccine site in > 97% of recipients, and inducing a wide spectrum of long-lasting humoral and cellular immune responses. The mechanisms behind inter-individual vaccine response variability are likely to involve host genetic variation, but have not been fully characterized. We report here the first smallpox vaccine-response genome-wide association study of over 1,000 recent recipients of Dryvax®. The data presented here focus on cellular immune responses as measured by both production of secreted IFNγ and quantitation of IFNγ secreting cells by ELISPOT assay. We identified multiple significant SNP associations in genes (RASA1, ADRA1D, TCF7L1, FAS) that are critical components of signaling pathways that directly control lymphocyte IFNγ production or cytotoxic T cell function. Similarly, we found many associations with SNPs located in genes integral to nerve cell function; findings that, given the complex interplay between the nervous and immune systems, deserve closer examination in follow-up studies.

Keywords

smallpox vaccine; vaccinia virus; genome-wide association study; single nucleotide polymorphism; interferon-gamma

Introduction

Although remarkably effective, the smallpox vaccine can result in serious, life-threatening adverse events (Fulginiti 2003; Fulginiti et al. 2003). This risk, as well as the large number

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Ethical Standards and Conflict of Interests

All experiments described here followed current, applicable U.S. laws. The authors have no conflicts of interest.

of contraindications (immunosuppression, organ transplants, HIV, heart conditions, skin diseases) in the current U.S. population, makes it difficult to prepare and deploy the vaccine in the face of bioterroristic threats. As stocks of the classical smallpox vaccines used during the eradication effort are dwindling, next-generation replacement vaccines are being researched, tested, and stockpiled (Artenstein and Grabenstein 2008). Smallpox vaccines are remarkably effective, inducing the characteristic pustule or “take” at the vaccine site in > 97% of recipients, and eliciting a wide spectrum of long-lasting immune responses, both humoral and cellular (Combadiere et al. 2004; Crotty et al. 2003; Frey et al. 2002; Hammarlund et al. 2003; Kennedy et al. 2009b). The mechanisms behind inter-individual vaccine response variability are currently unknown, but are likely to involve genetic variation in both the host and the virus. Given renewed interest in biodefense preparations to counter potential use of smallpox poxviruses as biological weapons, and to protect against emerging poxviruses such as monkeypox, it is important to understand how host genetic polymorphisms affect immune responses to smallpox vaccine. To date there have only been a few smallpox vaccine immunogenetic studies. A candidate gene study by Stanley et al. identified SNPs in cytokine genes associated with fever, and another candidate gene study by Reif et al. focused on genetic associations with adverse events after vaccination (Crowe 2007; Reif et al. 2008; Stanley et al. 2007). We have also reported candidate gene association studies examining the role of HLA genotypes in adaptive responses to smallpox vaccine (Ovsyannikova et al. 2011) and identifying IL18 and IL18R as loci involved in humoral immunity to smallpox vaccine (Haralambieva et al. 2011).

In this study we recruited a cohort of more than 1,000 recent smallpox vaccine recipients and conducted genome-wide SNP typing and a detailed examination of their cellular immune responses. To our knowledge, the data presented here are the first reported findings of a genome-wide association study on smallpox vaccine responses.

Materials and Methods

Subject Recruitment

The subjects described in this report came from a previously described study cohort consisted of 1,076 healthy subjects. Inclusion criteria included: 1) having received one, and only one, dose of Dryvax® smallpox vaccine within the four years prior to enrollment, 2) have a documented vaccine “take” indicating successful immunization (no additional information regarding vaccine take was available), 3) have never received a prior smallpox vaccination, 4) been in good general health at the time of the blood draw. These subjects (ages 18-40 at enrollment) had received the smallpox immunization as part of the civilian healthcare worker immunization program at Mayo Clinic in Rochester, MN, or the smallpox immunization program involving armed forces personnel at the Naval Health Research Center (NHRC) in San Diego, CA (Kennedy et al. 2009a) (Haralambieva et al. 2011; Ovsyannikova et al. 2011). The Institutional Review Boards of the Mayo Clinic and NHRC approved the study and written informed consent was obtained from each subject. Demographic information regarding this cohort has been previously published (Kennedy et al. 2009c).

Isolation of peripheral blood mononuclear cells (PBMC)

100 mL of whole blood was collected from each subject and PBMC were isolated within 24 hours by density gradient centrifugation according to standard protocols as previously described (Ryan et al. 2009). Isolated PBMC were resuspended at a concentration of 1×10^7 cells/mL in RPMI 1640 media containing l-Glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% dimethyl sulfoxide (Protide Pharmaceuticals, St. Paul, MN) and 20% fetal bovine serum (FBS; Hyclone, Logan, UT), frozen overnight at − 80°C and transferred to liquid nitrogen for long-term storage.

PBMC aliquots were thawed and rested as previously described (Ovsyannikova et al. 2005; Ryan et al. 2009). Briefly, cells were thawed and incubated overnight in culture medium containing 50 IU/ml of IL-2 (Proleukin®, Chiron, Emeryville, CA). Cells were then collected, washed, and resuspended in culture medium at a concentration of 2×10^6 cells/mL for use in both the ELISPOT and ELISA assays described below.

SNP typing and QC

DNA was extracted from whole blood or blood clots using the Gentra Puregene Blood kit (Gentra Systems Inc., Minneapolis, MN) and quantified by Picogreen (Molecular Probes, Carlsbad, CA). High density SNP analysis was performed using the Infinium HumanHap550 BeadChip array (Illumina, San Diego, CA) for the self-declared Caucasian subjects (n=580) and the 650K Infinium HumanHap650Y SNP BeadChip array for the subjects indicating their race as either African-American or unknown (n=217). DNA samples underwent amplification, fragmentation and hybridization onto each BeadChip, which were imaged on an Illumina BeadArray reader. Genotype calls based on clustering of the raw intensity data were made using BeadStudio 2 software. The resulting genotype data on SNPs were exported into SAS for analysis. Quality-control checks included genotyping reproducibility, gender checks, SNP and subject call-rate cutoffs of > 0.95, elimination of monomorphic SNPs, and a Hardy-Weinberg Equilibrium (HWE) check (SNPs with $p<1e^{-8}$ were flagged as having poor genotyping quality). This test for HWE accounted for the fact that subjects were recruited from two distinct racial groups using a large sample approximation to the stratified test of Schaid, et al (Schaid et al. 2006).

ELISPOT Assays

Total human IFN-γ and $CD8^+$ IFNγ ELISPOT assays (R & D Systems, Minneapolis, MN) were performed using PBMC cultures as previously described (Ovsyannikova et al. 2011; Ryan et al. 2005). Briefly, cell cultures were stimulated with inactivated vaccinia virus at an MOI of 5 for 24 hours. Three stimulated and three unstimulated replicate wells were set up for each subject. A single well containing PHA (5μg/ml) was used as a positive control. After a 24-hr incubation at 37°C in 5 % CO_2, each plate was washed and incubated with biotinylated detection antibody followed by chromogenic substrate according to the manufacturer's specifications. Plate scanning and spot counting on each plate was performed on an ImmunoSpot® S4 Pro Analyzer (Cellular Technology Ltd., Cleveland, OH) equipped with ImmunoSpot® version 4.0 software (Cellular Technology Ltd.). Counting parameters were consistent across all plates. Results are presented in spot-forming units (SFU) per well (200,000 cells).

IFNγ ELISA Assay

PBMCs were placed in 96-well at 2×10^5 cells per well. Experimental conditions for each subject were as follows: a PHA (5ug/ml) positive control, triplicate wells containing culture medium (unstimulated wells), and triplicate wells containing inactivated vaccinia virus (stimulated wells). Inactivated vaccinia virus (NYCBOH strain) was added at an MOI of 0.05 for four days following optimized procedures as described (Earl et al. 2001; Kennedy et al. 2009a; Ryan et al. 2009).

Commercial ELISA-based kits (BD Pharmingen) were used to detect IFNγ from culture supernatants according to the manufacturer's instructions. Reference standards, included in each assay, were used to calculate the concentration of each cytokine.

Principal Components Analysis of Race and Ethnicity

The sampling plan for this study called for the enrollment of both Caucasian and African-Americans. We identified 22,863 SNPs in low linkage disequilibrium with one another that spanned the genome at regular intervals with a density of less than one SNP per 100kb. We used these SNPs in the EIGENSOFT software package (http://www.hsph.harvard.edu) to assess population structure both across and within racial groups (Price et al. 2006). In addition to using the Eigenstrat axes of variation to describe population genetic structure, we used the genotype data to assign racial groups via a clustering procedure using the Structure program (Pritchard et al. 2000). The Eigenstrat axes of variation were also used to adjust for population stratification within each racial group.

Statistical analyses

Assessment of cytokine secretion and ELISPOT levels included data from the triplicate unstimulated wells and the triplicate vaccinia-stimulated wells as previously described (Ryan et al. 2009). The data were condensed to a single response measurement per individual; the difference between the median of the three unstimulated and the median of the three stimulated values. Data summaries were subsequently made using frequencies and percents for categorical variables, while both medians and inter-quartile ranges (IQRs) were utilized for continuous variables.

Associations between the two IFN-γ measures and the SNP genotypes were formally evaluated using linear regression models. Within the Caucasian and the African-American genetic subgroups we performed separate analyses for each outcome. The primary analyses of the SNPs assessed the evidence for an additive genetic effect on the outcome of interest. In contrast to the descriptive analyses, formal evaluations used repeated measures analyses to simultaneously model the multiple ELISPOT measures per subject. Recognizing that correlations likely exist across the multiple measures within an individual, we used generalized estimating equations (GEEs) to build this correlational structure into our models. Associations comparing stimulation-induced differences in cytokine secretion or ELISPOT values with SNPs were evaluated by testing the significance of the SNP by stimulation status interaction. These repeated measures models are analogous to paired t-tests, as they compare differences between the stimulated and unstimulated states within each individual, but the results are aggregated among all subjects with genotype and

phenotype data. Statistical analyses were performed after adjusting for the following set of demographic and clinical variables: gender; 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 (frozen or ambient); time of year when the sample was shipped (warm-weather months April-September vs. cold-weather months October-March); and the first four Eigenstrat axes of variation. In addition, associations of SNP genotypes with ELISPOT values included assay operator as a covariate due to systematic differences observed for these measures across the different operators. Skewness in the measured outcomes was corrected using an inverse cumulative normal (probit) transformation. Quantile-quantile (q-q) plots were generated to compare the observed distribution of p-values from all SNPs for each outcome to the expected distribution under the null hypothesis of no association. Lambda values that measure the inflation of significance due to population stratification or other causes were extracted and used to correct the observed p-value distributions when necessary. Statistical tests were performed using two-sided significance levels and primary analyses were performed using the R software package (Team 2008). All significant SNP associations are shown in Tables 1-6, however, several of these SNPs had very few subjects that were homozygous for the minor allele. In order to determine if the results from these individuals exerted undue influence on the overall association, their data were combined with that from subjects with one copy of the minor allele, and an additional series of models were run with the same adjusting factors as the ordinal model. The resulting estimates from these sensitivity analyses were compared to the per-allele estimates from the original analyses to verify their similarity. The SNPs are marked with an asterisk next to the rsid number in each table. The table legend contains the sensitivity p-values for each of these SNPs.

Results

SNP Typing Results

All individuals were typed using either the Illumina Infinium HumanHap550 or 650Y BeadChip arrays. Initial QC steps were used to remove suspect SNPs such as those with poor clustering, monomorphic SNPs, or those that failed HWE. Seventy-one subjects with low quality genotyping (call rates < 95%) were also excluded. Overall genotype concordance was 97.9% (99.9% when excluding non-called genotypes) indicating good reproducibility. PLINK genome-wide association study (GWAS) software was used to compute the inbreeding coefficients on the × chromosome for all subjects. This assessment identified three individuals who appeared female, but reported male gender on the study intake forms. Further analysis of the genotyping data indicated call rates for two of the subjects were low, making this assessment inconclusive. The third individual was found to be an XXY male with Klinefelter’s syndrome. Pair-wise identity by descent metric was used to measure the degree of relatedness between individuals and identified 22 pairs of individuals whose genotypes were consistent with being siblings. In addition, three pairs of individuals appeared to be genetically identical or genetically similar; both individuals from one of these pairs were excluded from analysis, as was one individual from each of the other two pairs. SNP call rates for the 550K beadchip ranged from 36.3% to 100%, with a mean call rate of 98.2% (650K beadchip call rates were similar). The SNPs (34,751 for 550K,

48,336 for 650K) with call rates < 95% were excluded from further analysis. We also assessed the call rates for each individual, which ranged from 50.5% to 99.98%, with an overall mean call rate of 98.6%. In addition, monomorphic SNPs (29 for 550K, 34 for 650K) were identified and excluded. HWE checks were performed and the 151 failing SNPs (714 for the 55K chip, 728 for the 650K beadchip) were removed from subsequent analysis. We also excluded SNPs for a given outcome ($CD8^+$ or total IFNγ ELISPOT) and racial cohort (Caucasian or African-American) if there were fewer than 10 observed minor alleles.

These initial QC checks resulted in a dataset for 1,000 subjects containing 525,972 SNPs for the Infinium HumanHap500 array and 611,820 SNPs for the Infinium HumanHap650Y array.

Population Stratification and Principal Components Analysis (PCA)

Upon recruitment, each subject's self-identified race and ethnicity was recorded. Our cohort consisted of 1,000 subjects with demographics as outlined in Table 1. Notably, 45 (4.2%) subjects were unsure of their ethnicity and 243 (22.6%) subjects did not list a race (having chosen: "More than one race", "Other", or "Don't Know"). Given the availability of high-density SNP data, we explored the use of PCA results to assign race and ethnicity to each individual (Figure 1). The genetic racial classifications were in perfect agreement with self-declared race for those who had self-declared as either African or Caucasian American (Table 1), and the genetic data classified 30, and 46, additional subjects as being of African, and Caucasian, descent, respectively. Because of the strong concordance between genetic and self-declared race among those with unambiguous self-declaration, and because the genetic classification captured additional subjects for analysis, we used the genetically defined racial groupings as we ran the GWAS separately by race.

Immune Outcomes

The cellular immune responses for each subject were quantified by IFNγ ELISPOT assays using both total PBMCs and $CD8^+$ purified T cells, and by measuring IFNγ secretion by ELISA. Each outcome was calculated by subtracting the median results in the unstimulated wells from the median results in the vaccinia-stimulated wells. After subtracting the background IFNγ response in each subject, our population (n=987) had a mean total IFNγ ELISPOT responses of 61.1 IFNγ spot-forming units/200,000 cells with an interquartile range of 24.0 to 88.0. For the $CD8^+$ IFNγ ELISPOT responses, our population (n=935) had a mean response of 15.2 IFNγ spot-forming units/200,000 cells with an interquartile range of −2.0 to 26.0. For both ELISA and ELISPOT results, negative numbers indicate that background levels of cytokine secretion or spot-forming cells were higher than the results from the wells containing virus-stimulated cells, perhaps due to viral-induced immune suppression. The larger IFNγ response seen with total PBMCs is an indicator of strong $CD4^+$ T cell and NK cell activity upon viral stimulation (Demkowicz et al. 1996; Mitra-Kaushik et al. 2007; Puissant-Lubrano et al. 2010). Table 1 shows the vaccinia-specific responses for each immune outcome for both the African American and Caucasian subjects. In the African American cohort, the total ELISPOT median(IQR) was: 46.0 (22.0-96.0) in the unstimulated wells and 112.0 (63.0-175.0) in the stimulated wells. For these same subjects the CD8 ELISPOT median(IQR) was 62.5 (27.5-107.0) unstimulated and 70.0

(31.5-120.5) stimulated, while the IFNγ ELISA median(IQR) was 268.9 (77.1-840.5) unstimulated and 687.6 (177.4-2,047.2) stimulated. In the Caucasian cohort, the total ELISPOT median(IQR) was: 48.0 (21.0-97.0) in the unstimulated wells and 121.0 (72.0-196.0) in the stimulated wells. For these same subjects the CD8 ELISPOT median(IQR) was 49.0 (23.0-99.5) unstimulated and 70.0 (37.5-126.0) stimulated, while the IFNγ ELISA median(IQR) was 211.2 (67.3-687.4) unstimulated and 639.2 (193.7-2,144.3) stimulated.

Genome Wide Analysis Results

The association analyses were conducted independently on the Caucasian and the African-American cohorts and are reported separately here. Although we are currently recruiting a second cohort of 1,000 vaccine recipients for true replication purposes, we conducted a preliminary comparison of the statistically significant SNPs in each race with the results from the other racial group.

Depicted in Figure 2 are the Q-Q plots for the two sub-cohorts (Caucasian and African-American) and the two outcomes (total and $CD8^+$ IFNγ ELISPOT). We observed a slight inflation of significance in the overall distribution of p-values, and therefore applied genomic control approaches to adjust for this inflation (inflation factor estimates for the African-American subjects are: 1.027 for the total and 1.151 for the $CD8^+$ ELISPOT outcomes, inflation factor estimates for the Caucasian subjects are: 1.019 for the total ELISPOT and 1.032 for the $CD8^+$ ELISPOT outcomes). All reported p-values are corrected for this genomic control inflation. In the resulting Q-Q plots, as expected, the majority of the corrected p-values fall on the slope line, with an upward trend at the low p-value end. Similarly, Figures 3 and 4 contain the Manhattan plots showing the chromosomal distribution and p-values of each SNP association with the indicated outcomes. Interesting regions, such as on chromosome 10 in Figure 4B, are discussed below.

Genetic Associations with $CD8^+$ T cell IFNγ ELISPOT responses

Our genome-wide analyses found evidence for multiple SNPs (n = 25) significantly associated with variations in cytotoxic T cell responses to smallpox vaccine as measured by $CD8^+$ IFNγ ELISPOT. Table 2 lists each of the SNPs in the Caucasian sub-cohort with a p-value $< 5.0 \times 10^{-7}$, while Table 3 lists the significant SNPs in the African-American sub-cohort.

The top associations for the Caucasian cohort were in: *PSD3* coding for the pleckstrin and Sec7 domain containing protein that is an exchange factor for ADP-ribosylation factor; *RASA1* coding for a cytoplasmic GTPase activating protein that inhibits the RAS-cyclic AMP pathway, thereby regulating cellular proliferation and differentiation (Friedman et al. 1993; Trahey et al. 1988); RGS6, a G protein signaling regulator (Chatterjee et al. 1997); a transfer RNA for the anticodon UUG (glutamine) (Nemoto et al. 1991); *SEPT11*, a filament-forming cytoskeletal GTPase (Hanai et al. 2004); *PLS3*, a member of the plastin actin binding family (Lin et al. 1993); *UTRN*, an actin-binding protein localized to neuromuscular junctions (Burton et al. 1999); a thioredoxin pseudogene, *AF357530*; *TCERG1L*, a

transcription elongation regulator protein; and the *ADRA1D* gene encoding for an adrenergic receptor controlling response to epinephrine (Bruno et al. 1991).

In the African-American cohort, variations in $CD8^+$ IFNγ ELISPOT responses were associated with: *TCF7L1* a transcription factor participating in the Wnt signaling pathway (Sagara and Katoh 2000); and a zinc-finger protein *ZNF613*.

Genetic Associations with Total IFNγ ELISPOT Responses

We also found SNPs (n=10) associated with total IFNγ ELISPOT responses as shown in Tables 4 and 5. None of the SNP associations reached genome-wide significance levels in the Caucasian cohort. In the African-American cohort, the top associations included: the DNA-binding transcription factor *NFIB* (Grunder et al. 2003); *FAS*, which mediates T cell induced cytotoxicity (Itoh et al. 1991); *KCNQ1*, a potassium channel gene located in the long QT-syndrome locus (Wang et al. 1996); an inhibitory G protein that downregulates adenylate cyclase activity, *GNAI1*(Sullivan et al. 1986); *TBXAS1*, a thromboxane A synthase (Yokoyama et al. 1991); the delta subunit of the cGMP specific phosphodiesterase, *PDE6D* (Florio et al. 1996); and *OPRM1*, the primary opioid receptor for both opioids and endogenous opioid peptides (Bond et al. 1998).

Genetic Associations with Secreted IFNγ

In addition to assessing cellular immunity by IFNγ ELISPOT, we also measured secreted IFNγ by ELISA assay. The Manhattan and Q-Q plots illustrating the SNP associations with this outcome are depicted in Figures 5 and 6. Top SNP associations (n=11) are listed in Tables 5 and 6. In the Caucasian cohort, we identified associations between IFNγ secretion and SNPs in: the protein phosphatase *PPM1H* gene; *DAOA* the D-amino acid oxidase activator gene that is also a susceptibility locus for schizophrenia and bipolar disorder (Chumakov et al. 2002); and *SORCS1*, a vacuolar protein sorting domain-containing receptor.

Analysis of the African-American cohort indicated associations between secreted IFNγ and: *HPSE2* an endoglycosidase involved in remodeling the extracellular matrix (McKenzie et al. 2000) (although our sensitivity analysis revealed that this association was likely driven by the single GG genotype individual with extremely low levels of IFNγ); the *SOX6* transcription factor (Cohen-Barak et al. 2001); a ubiquitin regulatory × domain containing protein *UBXD 3*(Schuberth and Buchberger 2008); *OR10G6*, an olfactory receptor G protein; and *AMICA1*, involved in leukocyte transmigration through epithelial/endothelial tissues.

When examining SNP associations across racial group we established the following criteria: a p-value of $< 5 \times 10^{-7}$ in one cohort; a p-value of < 0.01 in the second cohort; and an associated effect on the immune outcome with the same directionality in both cohorts (e.g. increased IFNγ response). None of the identified SNPs in Tables 2 and 3 fulfilled all three requirements, nor did any of the SNP associations with total IFNγ ELISPOT in Tables 4 or 5 meet these requirements. Interestingly, rs7987983 (*DAOA* gene) met each of our validation requirements, being significantly associated with a similar effect in both Caucasians and

African-Americans. Our data suggest that there is no dominant allele controlling immune responses to smallpox vaccine. Given the complex, multigenic nature of immune responses we are not surprised that diverse genetic loci show significant phenotype associations in different racial groups.

Discussion

Overview of results

Our study population had widely varying levels of cellular immune markers to vaccinia virus as measured by IFNγ ELISPOT responses of both total PBMCs and $CD8^+$ T cells to viral stimulation. One of the advantages of the genome-wide approach over candidate gene studies is the unbiased assessment of SNP associations across the entire genome, with the ability to discover novel genes involved in immune function. In fact, our genome-wide analysis identified both immune-related genes as well as genes involved in neuronal cell signaling and transcriptional activity. Although these novel genes have not yet been linked to immune functions, many of the key pathways they control in neuronal cells are used by lymphocyte lineages to control cell growth, activation, and differentiation.

$CD8^+$ IFNγ ELISPOT responses in our Caucasian populations ranged from −102 to 238 spot-forming units/200,000 cells and variations were associated with several genes (Table 2) that are components of critical signaling pathways in immune cells including: RASA1 and ADRA1D. Ras activation is essential for T and B cell development, activation, and function (Ehrhardt et al. 2002) and the RASA1 gene encodes for a protein that stimulates GTPase activity of RAS p21, thereby suppressing RAS function. Mutations in RASA1 can alter this regulatory activity and are associated with basal cell carcinoma (Friedman et al. 1993; Hu et al. 2009) and capillary malformation (Eerola et al. 2003). Similarly, ADRA1D is a G protein-coupled receptor that signals through the Gαq subunit, which leads to activation of phospholipase C and Rho. Although ADRA1D is primarily involved in hormone signaling in the nervous system, the same downstream signaling pathways it activates are crucial to lymphocyte activation development, differentiation, activation, and migration (Tybulewicz and Henderson 2009). ADRA1D can also transactivate EGF receptors with subsequent induction of p42/p44 MAPK signaling (Chen et al. 2006).

In the African-American population, variations in $CD8^+$ IFNγ ELISPOT responses (Table 3) were associated with rsrs6737773 in TCF7L1. TCF7L1 is a transcription factor activated by the wnt signaling pathway. It has recently been shown that wnt/TCF-1 is required for the expansion of primary/memory cytotoxic T lymphocytes as $TCF7^{-/-}$ mice have reduced primary CD8 responses and impaired memory T cell memory (Jeannet et al. 2010; Zhou et al. 2010). It has also been shown that simultaneous stimulation of the TCR and TCF-1/β-catenin pathways favors the development of memory $CD8^+$ T cells (Goldrath et al. 2000; Zhao et al. 2010). Polymorphisms altering the expression or function of this transcription factor may lead to increased/decreased T cell survival and maintenance, thereby affecting antigen-specific, IFNγ T cell responses.

None of the SNPs demonstrated significant associations with total IFNγ ELISPOT responses in the Caucasian cohort. As it is unlikely that genetic polymorphisms have no effect on

immune responses, we may have lacked sufficient power to detect associations, especially if the individual contribution of each SNP is small. Alternatively, this assay measures both antigen-specific IFNγ secretion by both CD4 and CD8 T cells, as well as non-antigen specific IFNγ release by NK cells and it is possible that different genetic elements influence IFNγ production in the different cell types making it difficult to pinpoint the various SNP associations. Follow-up studies on specific cell subsets will be required to investigate this further.

We did find several interesting associations with total IFNγ ELISPOT outcomes in the African-American cohort (Table 4). Rs7860845, an intronic SNP in *NFIB*, was associated with lower total IFNγ ELISPOT levels in this population. *NFIB* is a member of the Nuclear Factor I family of DNA binding proteins that regulate the transcription of multiple cellular signaling pathways (insulin, TGF-β, TNFa, steroid hormones, vitamins), and are essential for the replication of many viruses (Gronostajski 2000). Interestingly, overexpression of NFIB2 leads to downregulation of surface CD4 expression (Sheeter et al. 2003). One potential mechanism behind the genetic association between NFIB and IFNγ response might be the control of CD4 expression and concomitant up/down-regulation of T helper responses upon antigen exposure. Three SNPs (rs9658691, rs3758483, rs983751) in the *FAS* gene were all associated with increases in total IFNγ ELISPOT response in African-Americans. *FAS* encodes a death receptor mediating T and NK cell cytotoxicity(Lowin et al. 1994), antigen induced cell death of T lymphocytes, apoptosis of FasL expressing B cells and APCs resulting in decreased antigen presentation, and maintenance of immune privilege in the brain and central nervous system (Bechmann et al. 1999). All of the above SNPs are in close LD ($r^2 > 0.89$, Figure 7) and show identical associations (Table 4) and likely tag a single causal SNP, which should be explored in future fine-mapping studies and/or functional studies. Polymorphisms affecting Fas expression and/or function may alter T cell survival, leading to differences in IFNγ production. Alternatively, genetic variations in the *FAS* gene may have downstream effects of MAPK or p38 pathways, which has been shown to affect IFNγ secretion (Rincon et al. 1998). An intronic SNP (rs2523194) in *GNAI1* was associated with decreased total IFNγ ELISPOT response. *GNAI1* is a G protein that modulates signaling cascades (c-SRC and MEK/ERK pathway, adenylate cyclase pathway) in response to receptor activation (IL-1, IL-8, CXCR4, CCR3, NGR-p75). *PDE6D* is a retinal rod rhodopsin-sensitive phosphodiesterase that degrades cGMP (Lorenz et al. 1998), but is also found in a variety of lymphoid tissues (Yanai et al. 2005). Related phosphodiesterases (PDE7A3, PDE9A1, PDE9A6) are known to influence immune cell activation (Omori and Kotera 2007), and it has been reported that signaling through PDE7 increases cytokine expression and proliferation (Li et al. 1999). Another significant association was found between rs1319339 in the mu opioid receptor *OPRM1* and increased IFNγ ELISPOT response in African-Americans. Opioid receptors are expressed on lymphoid cells (Chuang et al. 1995), and control expression of both cytokines and cytokine receptors (Finley et al. 2008), including the production of IFNγ (Brown and Van Epps 1986; Lysle et al. 1993; Wang et al. 2001).

Our association analysis with secreted IFNγ revealed many significant associations. For the Caucasian subjects these included: rs3847906 in the recently identified PP2C family

member PPM1H, which alters proliferation and apoptosis signals (Behrens et al. 2003; Sugiura et al. 2008). In the African-American cohort significant associations were found in several genes with no currently known immunologic functions: rs1437635 in the *SOX6* gene; rs3179690 in the *UBXD3* gene; and rs1453654 in the *OR10G6* gene, a G-protein coupled receptor involved in odor perception (Malnic et al. 2004). Rs11216816 is located in *AMICA1*, which encodes for a costimulatory molecule on γδ T cells that stimulates cellular proliferation and cytokine production (Witherden et al. 2010).

Comparison of the race-specific analyses can be viewed as a form of replication for those SNPs; statistically significant and consistent results for the same SNP across different racial groups provides greater confidence of a genetic effect on the cytokine in question. Furthermore, replication of effect across genetically distinct groups expands the scope of inference for a given association. However, we have previously reported that gender and race affect smallpox vaccine outcomes (Kennedy et al. 2009c) and genetic control of vaccine responses is likely a complex, multigenic phenomenon confounded by racial effects, making validation in a different racial cohort a high bar to reach. Thus, it is not surprising that only a single SNP met these validation requirements: rs7987983, which is near to *DAOA*, is associated with decreased IFNγ secretion. A nearby SNP ($r^2 = 0.85$ in our Caucasian cohort; $r^2 = 0.68$ in our African-Americans), rs7337090 was also associated with the same effect but missed our p-value cutoff, having a p-value of 7.01×10^{-7} in the Caucasian cohort (See Figure 1). DAOA is a D-amino acid oxidase activator, and polymorphisms in this gene have been associated with susceptibility to bipolar disorder (Hattori et al. 2003) and to schizophrenia (Chumakov et al. 2002), but there are no existing data indicating a role for this gene in immune function.

A strength of our study is the genome-wide range covered by the Illumina HumanHap SNP chips, allowing us to examine not only known immune genes, but also to identify associations between immune response and additional genes involved in immune function and regulation. Our study is also the largest genotype-phenotype study examining the immunogenetics of smallpox vaccine response to date. Our results have pinpointed many directions for future studies for furthering our understanding of the genetic control of smallpox vaccine responses.

Many of the genes for which we found significant associations (*RASA1*, *OPRM1*, *GNAI1* and *TCF7L1*) are critical components of pathways that directly control lymphocyte cytokine production. These associations with IFNγ responses to smallpox vaccine are therefore biologically plausible, providing corroborative evidence for their significance. We have also found associations between IFNγ responses and genes not known to have direct immune function (*ZNF613*, *ADRA1D*, *UTRN*, *DAOA*, *SOX6*) or SNPs located in gene deserts or pseudogenes (LOC100128245). The biologic plausibility of these results is difficult to explain as they may represent additional, unsuspected functions of these genes in lymphoid populations, SNPs in LD with true causal SNPs, or even false positives. Given the cross-talk between both the nervous and immune systems, it is intriguing that so many of the significant associations with IFNγ outcomes were found with SNPs in genes integral to nerve cell activity (Elenkov et al. 2000). A limitation of this report is the lack of true replication in a similar cohort. We have previously identified racial differences in smallpox

vaccine-induced immune responses, and the use of two different races for validation is therefore not optimal. Given the high rate of false-positive results with GWAS studies, replication of these findings in an independent cohort of the same racial background is currently underway. This will be an important next step toward validating the results. Furthermore, functional studies designed to elucidate the mechanisms behind the associations could reveal novel means of immune regulation and control.

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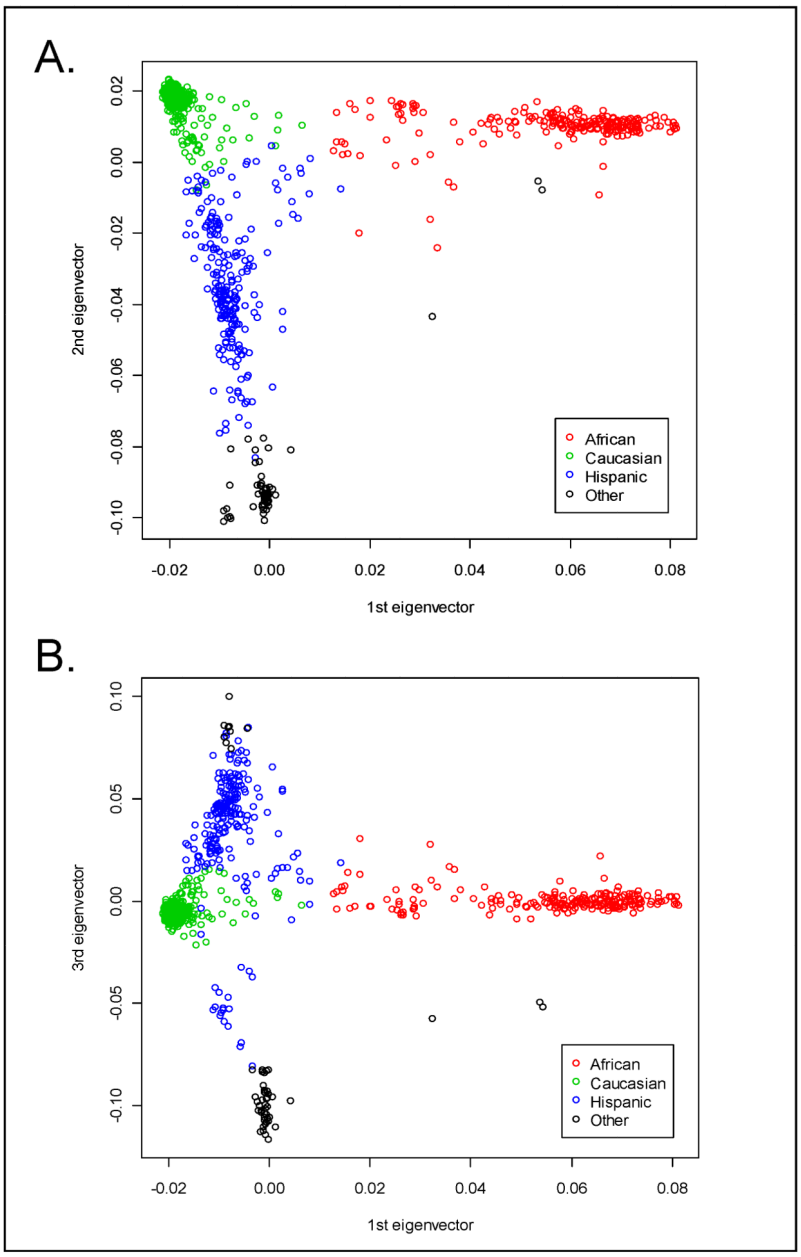

Figure 1. Plots of genetic similarity according to Eigenstrat-derived PCA-based axes of genetic variation

Highlighted clusters represent racial/ethnic groupings that are consistent with self-declared racial or ethnic group (Red = African-American, Green = Caucasian, Blue = Hispanic, Black = Other). The axes represent the eigenvectors explaining the greatest amount of average genetic sharing using data from 22,863 independent SNPs.

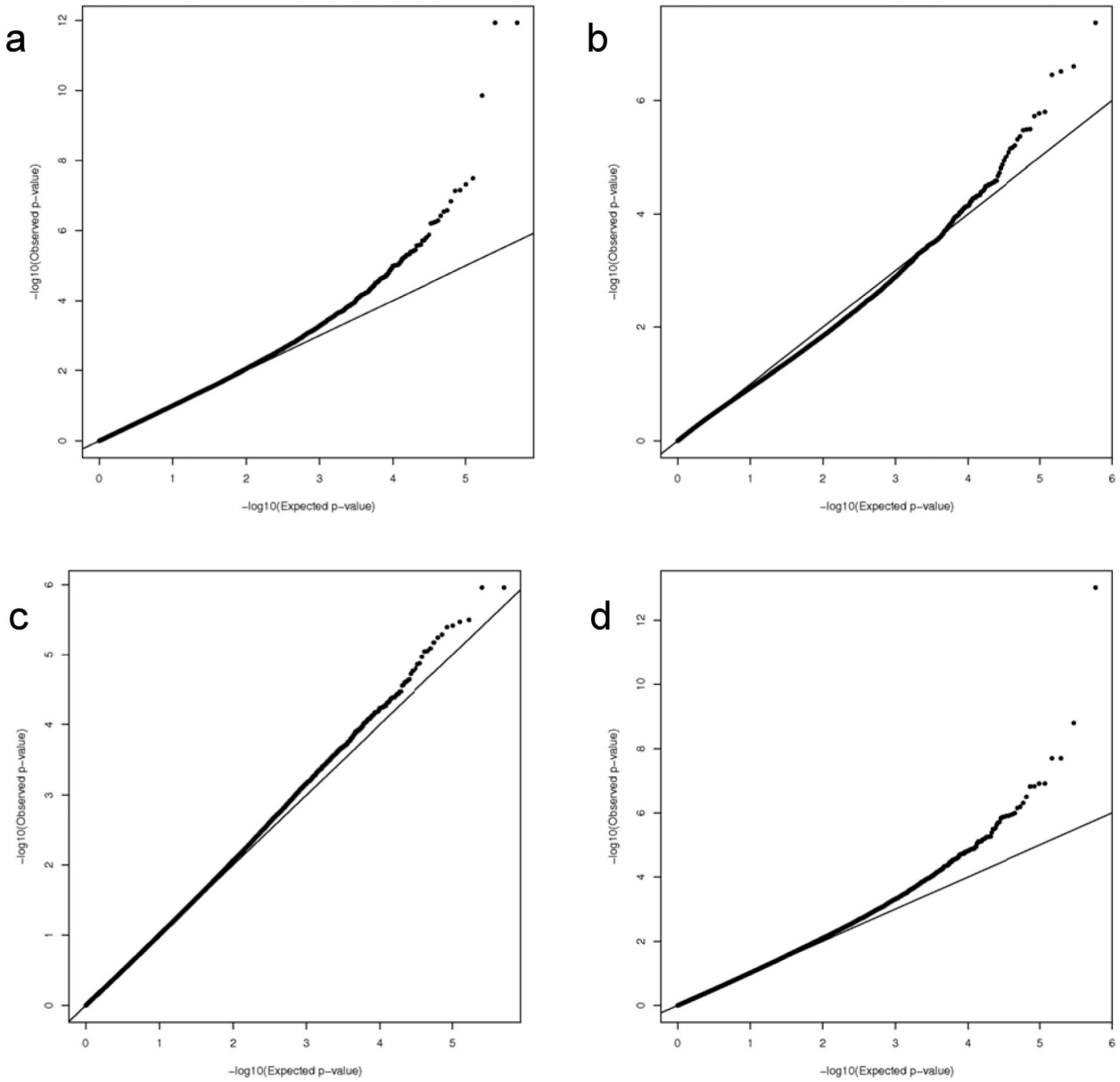

Figure 2. Quantile-Quantile plots of the expected (x-axis) and observed (y-axis) −log10(p-value) in our smallpox vaccine response GWAS

A) Results for the Caucasian cohort and $CD8^+$ IFNγ ELISPOT outcome. B) Results for the African-American cohort and CD8 IFNγ ELISPOT outcome. C) Results for the Caucasian cohort and total IFNγ ELISPOT outcome. D) Results for the African-American cohort and total IFNγ ELISPOT outcome.

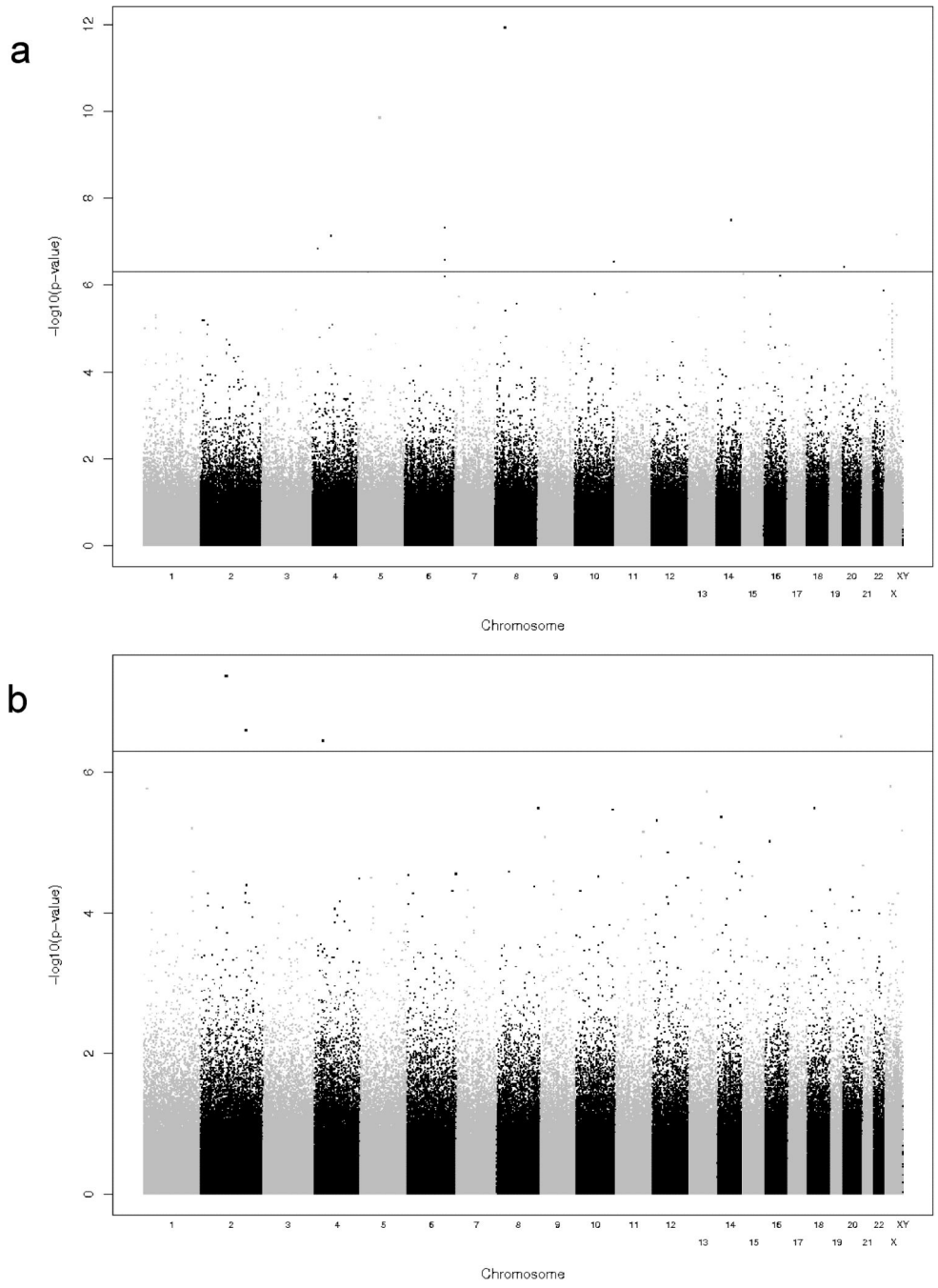

Figure 3. Summary of GWAS results for cellular immune responses as measured by CD8$^+$ IFNγ ELISPOT

The y-axis displays the –log10 of the p-value for each SNP association and the x-axis displays the chromosomes in alternating black and gray. P-values were adjusted for demographic and clinical variables as described in the Methods. A) Results for Caucasian cohort. B) Results for African-American cohort.

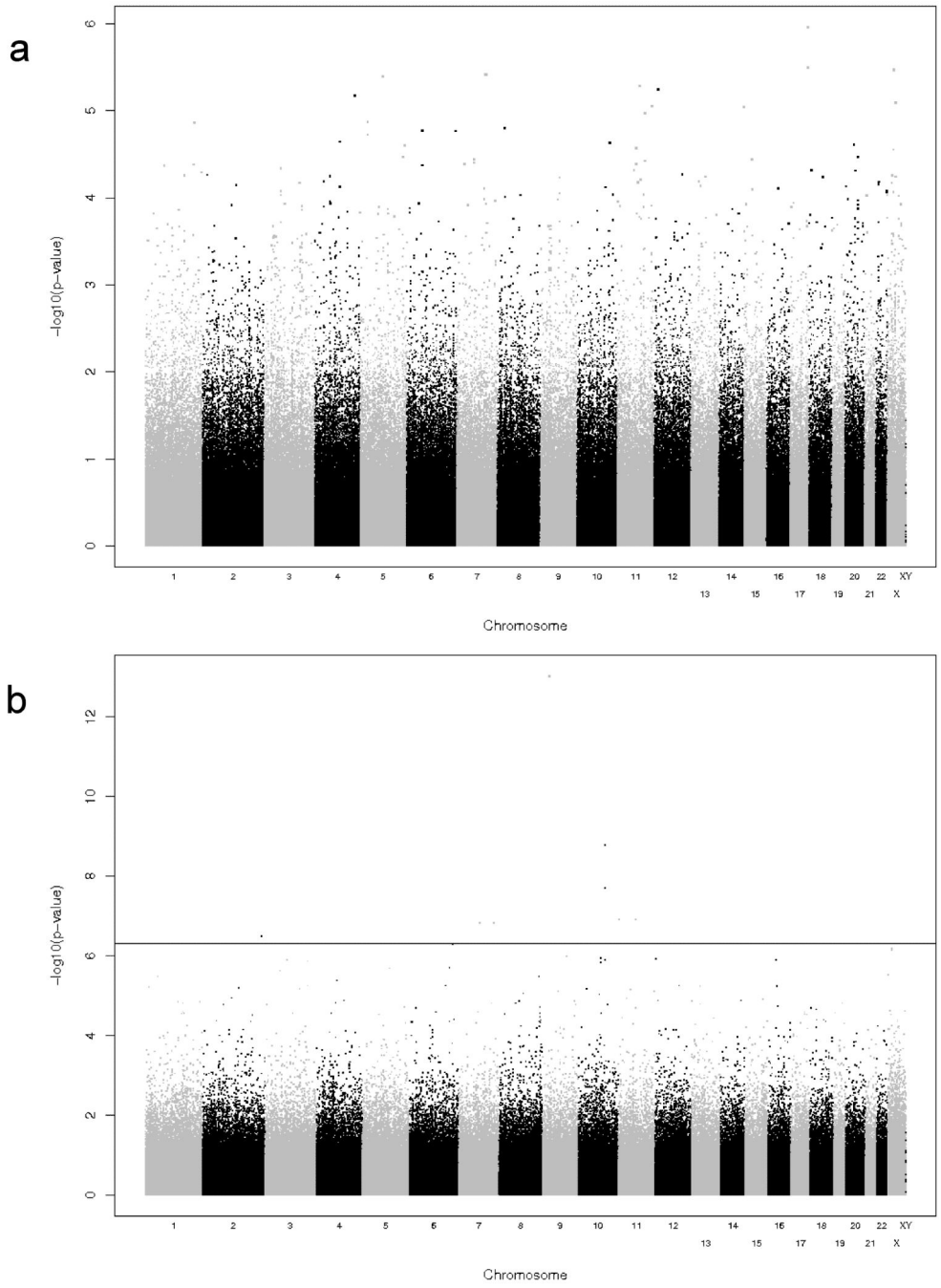

Figure 4. Summary of GWAS results for cellular immune responses as measured by Total IFNγ ELISPOT

The y-axis displays the −log10 of the p-value for each SNP association (please note that the scale on the two plots are not equal) and the x-axis displays the chromosomes in alternating black and gray. P-values were adjusted for demographic and clinical variables as described in the Methods. A) Results for Caucasian cohort. B) Results for African-American cohort.

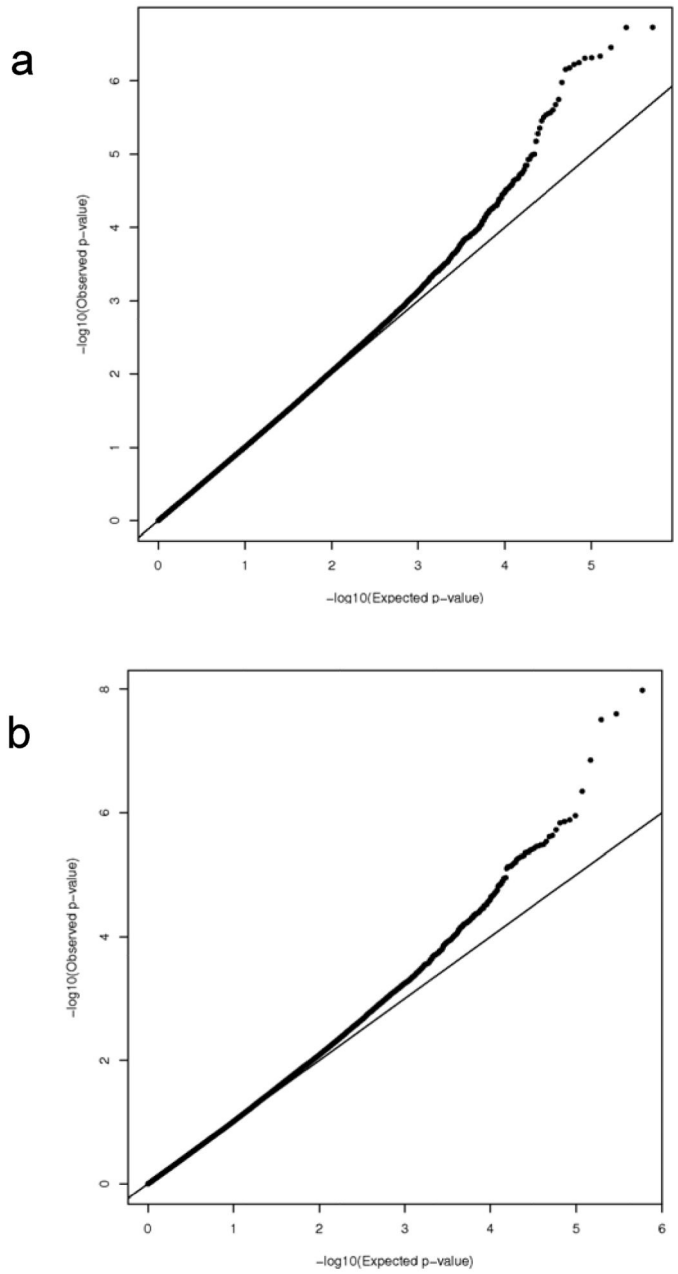

Figure 5. Quantile-Quantile plots of the expected (x-axis) and observed (y-axis) −log10(p-value) for secreted IFNγ response

A) Results for the Caucasian cohort. B) Results for the African-American cohort.

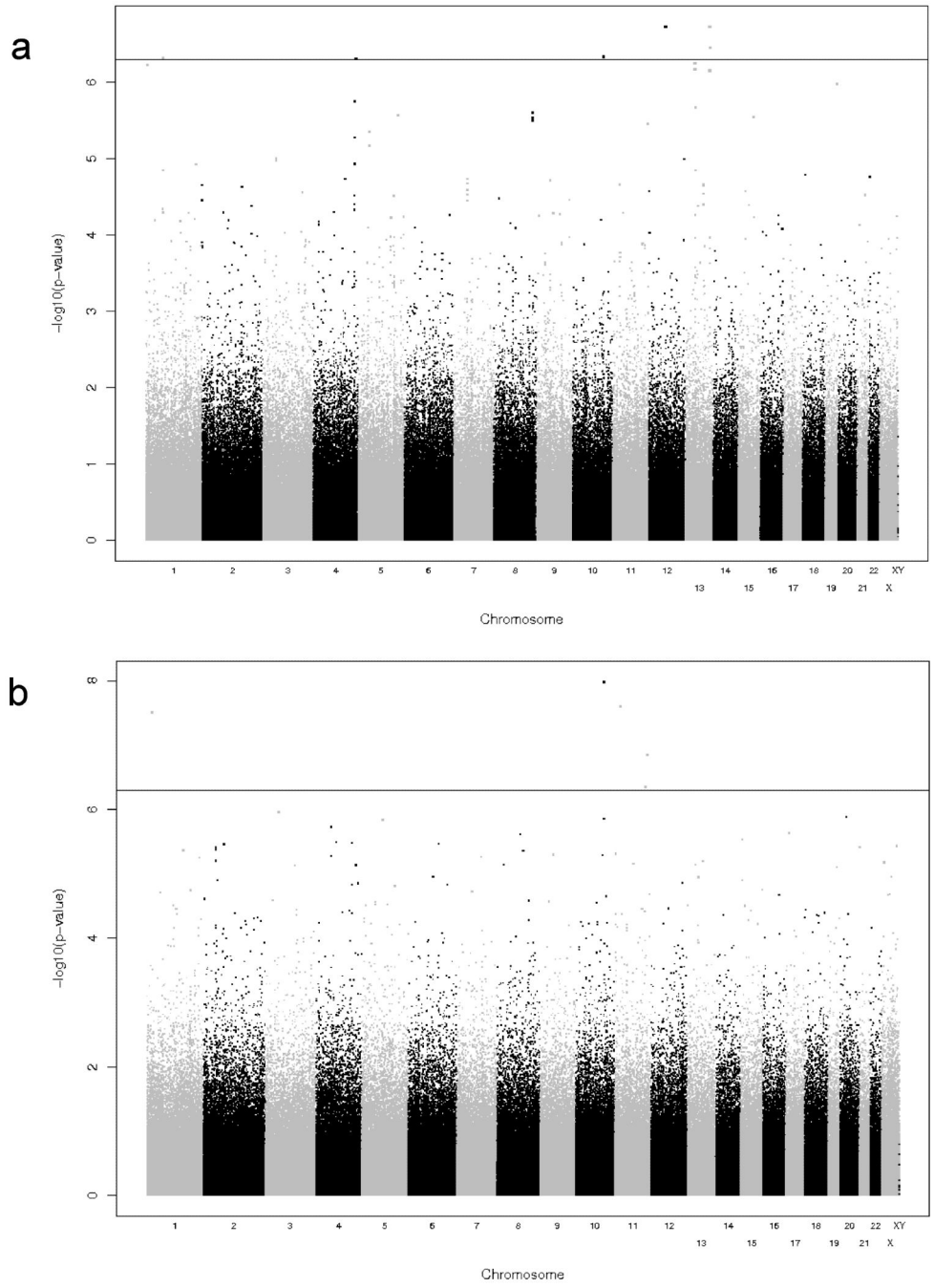

Figure 6. Summary of GWAS results for cellular immune responses as measured by secreted IFNγ

The y-axis displays the −log10 of the p-value for each SNP association (please note that the scale on the two plots are not equal) and the x-axis displays the chromosomes in alternating black and gray. P-values were adjusted for demographic and clinical variables as described in the Methods. A) Results for Caucasian cohort. B) Results for African-American cohort.

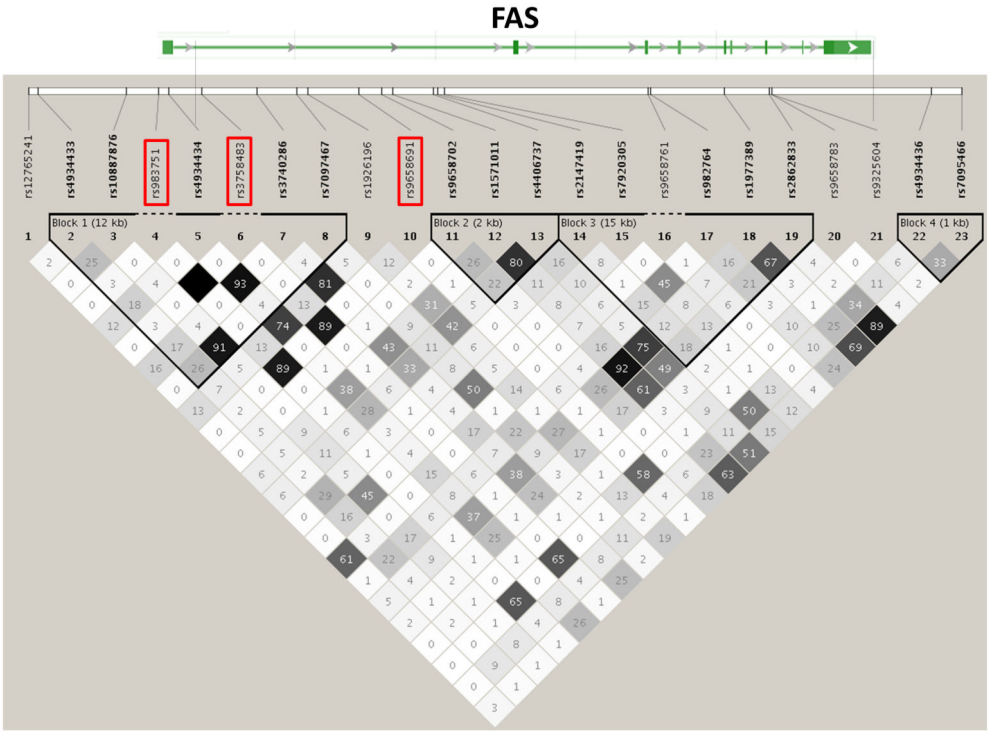

Figure 7. Genetic region containing FAS and 3 SNPs significantly associated with variations in total IFNγ ELISPOT outcomes in the African-American cohort

The top bar represents the exon/intron structure of the indicated gene. Vertical bars match the location of the SNPs at the top of the LD plot. The r^2 relationship between each pair of SNPs is indicated by the color and number of diamonds in the LD plot. LD blocks (Gabriel definition) are represented by the bold triangles. SNPs showing significant associations in this study are highlighted in boxes.

Table 1

Racial breakdown of study cohort and immune outcome summaries

Columns indicate the racial assignment by principal components analysis (PCA). This analysis used the genotyping data from the HumanHap550 BeadChip and the 650K Infinium HumanHap650Y SNP BeadChip to assign a genetically determined race to each subject. Self reported race is indicated in the top section of the table. Summary immune outcomes for each racial subcohort are indicated in the next rows of the table.

	African-American		Caucasian-American	
	N	**%**	**N**	**%**
Self-Declared Race				
African American	177	86	0	0.0
Caucasian	0	0.0	486	91
More than one race	17	8	32	6
Other or do not know	13	6	14	3
	Mean	**St. Dev.**	**Mean**	**St. Dev.**
Total ELISPOT (SFU/200,000 cells)	60.2	60.0	66.6	60.9
	Median	**Q1-Q3**	**Median**	**Q1-Q3**
	51	23-84	56	28-95
	Mean	**St. Dev.**	**Mean**	**St. Dev.**
CD8 T cell ELISPOT (SFU/200,000 cells)	9.2	38.2	17.9	35.2
	Median	**Q1-Q3**	**Median**	**Q1-Q3**
	4	−7-17.5	12	0-32
	Mean	**St. Dev.**	**Mean**	**St. Dev.**
IFNγ ELISA (pg/ml)	909.9	1685.1	853.3	1621.1
	Median	**Q1-Q3**	**Median**	**Q1-Q3**
	271.1	12.1-1224.7	310.7	28.3-1305.6

Table 2

SNPs showing significant association with CD8$^+$ IFNγ ELISPOT in the Caucasian cohort

SNP ID[a]	Chromosome[b]	Gene[c]	Location[d]	Distance from Gene	MAF[e]	Genotype	N[f]	Median (IQR)[g]	p-value[h]
rs1110820	8	PSD3	3′downstream	24512	10	GG	472	13 (0,34.3)	1.15E-12
						GA	10	−2 (−5,6.3)	
						AA	0	(,)	
rs4921930	8	PSD3	3′downstream	28916	10	AA	472	13 (0,34.3)	1.15E-12
						AG	10	−2 (−5,6.3)	
						GG	0	(,)	
rs6890495	5	RASA1	5′upstream	39836	13	GG	469	13 (0,35)	1.39E-10
						GA	13	2 (−12,9)	
						AA	0	(,)	
rs10138587	14	RGS6	5′upstream	49574	13	GG	468	13 (0,34.3)	3.18E-08
						GA	13	0 (−1,11)	
						AA	0	(,)	
rs6570670	6	TRNAQ-UUG	5′upstream	136541	16	AA	464	13 (0,35)	4.74E-08
						AC	16	−2 (−11.8,9.3)	
						CC	0	(,)	
rs10218219	X	PLS3	intron	0	18	GG	455	13 (0,33.5)	6.92E-08
						GA	18	3.5 (−6.8,10.5)	
						AA	0	(,)	
rs2645668	4	SEPT11	intron	0	26	AA	449	13 (0,35)	7.25E-08
						AG	26	1.8 (−12.3,8.8)	
						GG	0	(,)	
		AF357530	5′upstream	9477	26	AA	449	13 (0,35)	7.25E-08
						AG	26	1.8 (−12.3,8.8)	
						GG	0	(,)	

SNP ID[a]	Chromosome[b]	Gene[c]	Location[d]	Distance from Gene	MAF[e]	Genotype	N[f]	Median (IQR)[g]	p-value[h]
rs16894201	4	LOC729006	3′downstream	34507	12	CC	470	13 (0,34.8)	1.45E-07
						CA	12	3.5 (−15.3,10.6)	
						AA	0	(,)	
rs1953793	6	UTRN	3′downstream	113196	15	AA	466	13 (0,34.8)	2.63E-07
						AG	15	2 (−2.5,9.5)	
						GG	0	(,)	
rs12764951*	10	TCERG1L	3′downstream	42865	11	GG	471	13 (0,34.5)	2.84E-07
						GA	9	1 (−2,6)	
						AA	1	−7 (−7,−7)	
rs6052456	20	ADRA1D	intron	0	220	GG	284	14.5 (1,41)	3.75E-07
						GA	166	10 (−2,29)	
						AA	27	5 (−2,15.5)	

[a] rs SNP identification number

[b] Chromosomal location of the indicated SNP

[c] Gene or genetic region containing the indicated SNP

[d] Location of the SNP relative to the gene

[e] Minor Allele Frequency

[f] Number of subjects with a given genotype

[g] Median outcome measurement for each genotype group. Results expressed as SFU/200,000 cells. The interquartile range is shown in parentheses

[h] P-values were adjusted for demographic and clinical variables as well as inflation of significance described in the Methods.

* Sensitivity analysis p-value for rs12764951 = 6.4E-05.

Table 3

SNPs showing significant association with CD8$^+$ IFNγ ELISPOT in the African-American cohort

SNP ID[a]	Chromosome[b]	Gene[c]	Location[d]	Distance from Gene	MAF[e]	Genotype	N[f]	Median (IQR)[g]	p-value[h]
rs6737773	2	TCF7L1	intron	0	10	AA	183	5 (−6,19)	4.27E-08
						AG	10	−12.5 (−22,−7)	
						GG	0	(,)	
rs16864122	2	LOC375295	intron	0	11	GG	177	5 (−6,19)	2.52E-07
						GA	11	−19 (−32.5,−9.5)	
						AA	0	(,)	
rs1549932	19	ZNF613	3′downstream	6244	13	AA	180	5 (−7,19)	3.08E-07
						AG	13	−6 (−7,1)	
						GG	0	(,)	
rs16880706*	4	LOC645641	3′downstream	335029	11	AA	182	5 (−7,19)	3.54E-07
						AG	9	−5 (−11,1)	
						GG	1	−31 (−31,−31)	

[a] rs SNP identification number

[b] Chromosomal location of the indicated SNP

[c] Gene or genetic region containing the indicated SNP

[d] Location of the SNP relative to the gene

[e] Minor Allele Frequency

[f] Number of subjects with a given genotype

[g] Median outcome measurement for each genotype group. Results expressed as SFU/200,000 cells. The interquartile range is shown in parentheses

[h] P-values were adjusted for demographic and clinical variables as well as inflation of significance described in the Methods.

* Sensitivity analysis p-value for rs16880706 = 5.43E-08.

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Table 4

SNPs showing significant association with total IFNγ ELISPOT in the African-American cohort

SNP ID[a]	Chromosome[b]	Gene[c]	Location[d]	Distance from Gene	MAF[e]	Genotype	N[f]	Median (IQR)[g]	p-value[h]
rs7860845*	9	NFIB	intron	0	12	AA	188	53 (23.8,85.3)	9.48E-14
						AG	10	18.5 (6.3,40.3)	
						GG	1	−30 (−30,−30)	
rs9658691*	10	FAS	intron	0	10	AA	191	48 (22,79.5)	1.63E-09
						AG	8	102.5 (81.8,169.3)	
						GG	1	233 (233,233)	
rs3758483*	10	FAS	5′upstream	1552	11	AA	190	48 (22,79.8)	1.97E-08
						AG	9	93 (64,168)	
						GG	1	233 (233,233)	
rs983751*	10	FAS	5′upstream	3595	11	CC	190	48 (22,79.8)	1.97E-08
						CA	9	93 (64,168)	
						AA	1	233 (233,233)	
rs2237875*	11	KCNQ1	intron	0	36	AA	167	54 (28.5,85.5)	1.21E-07
						AG	30	28 (0,52.5)	
						GG	3	−9 (−12,−2)	
rs11237722	11	C11orf	5′upstream	4536	12	GG	188	48 (21.8,81.8)	1.21E-07
						GA	12	81 (54.5,130.3)	
						AA	0	(,)	
rs2523194	7	GNAI1	intron	0	12	AA	188	53 (23.8,85.3)	1.48E-07
						AG	12	18.5 (−9.5,36)	
						GG	0	(,)	
rs17161201	7	TBXAS1	intron	0	10	GG	189	52 (24,85)	1.50E-07
						GA	10	15.5 (−5.8,54.3)	
						AA	0	(,)	

SNP ID[a]	Chromosome[b]	Gene[c]	Location[d]	Distance from Gene	MAF[e]	Genotype	N[f]	Median (IQR)[g]	p-value[h]
rs4973479	2	PDE6D	intron	0	10	GG	190	52.5 (24,85)	3.15E-07
						GA	10	19.5 (−11.5,25.8)	
						AA	0	(,)	
rs1319339	6	OPRM1	5′upstream	29259	15	AA	185	48 (21,81)	4.91E-07
						AG	15	80 (63.5,127.5)	
						GG	0	(,)	

[a] rs SNP identification number

[b] Chromosomal location of the indicated SNP

[c] Gene or genetic region containing the indicated SNP

[d] Location of the SNP relative to the gene

[e] Minor Allele Frequency

[f] Number of subjects with a given genotype

[g] Median outcome measurement for each genotype group. Results expressed as SFU/200,000 cells. The interquartile range is shown in parentheses

[h] P-values were adjusted for demographic and clinical variables as well as inflation of significance described in the Methods.

[*] Sensitivity analysis p-values are as follows: rs7860845 = 2.40E-09, rs9658691 = 1.18E-08, rs3758483/rs983751 = 9.09E-07, rs2237875 = 1.12E-05

Table 5

SNPs showing significant association with secreted IFNγ in the Caucasian cohort

SNP ID[a]	Chromosome[b]	Gene[c]	Location[d]	Distance from Gene	MAF[e]	Genotype	N[f]	Median (IQR)[g]	p-value[h]
rs3847906	12	PPM1H	intron	0	12	AA	482	304.3 (31.4,1334.4)	1.86E-07
						AG	12	66.4 (−397.3,472.6)	
						GG	0	(,)	
rs7987983	13	DAOA	5′upstream	1027284	225	GG	293	382 (58,1373.1)	1.87E-07
						GA	177	193.2 (−7.3,1319.9)	
						AA	24	37 (−24.7,131)	
rs16968401	13	LOC341604	5′upstream	136323	10	GG	487	315.1 (24.9,1316.1)	3.50E-07
						GA	10	72.6 (21.9,243.7)	
						AA	0	(,)	
rs10458732	10	SORCS1	intron	0	10	GG	488	313.7 (31.6,1324.8)	4.61E-07
						GA	10	60.7 (−12.4,484.1)	
						AA	0	(,)	
rs11209457	1	LOC100133218	3′downstream	177802	42	GG	455	331.3 (42.2,1376.2)	4.85E-07
						GA	42	9.1 (−166.4,425.5)	
						AA	0	(,)	
rs1588571	4	LOC100129666	3′downstream	2482	18	GG	474	322 (33.7,1308.9)	4.92E-07
						GA	18	8.9 (−218.4,178.6)	
						AA	0	(,)	

[a] rs SNP identification number

[b] Chromosomal location of the indicated SNP

[c] Gene or genetic region containing the indicated SNP

[d] Location of the SNP relative to the gene

[e] Minor Allele Frequency

[f] Number of subjects with a given genotype

[g] Median outcome measurement for each genotype group. Results expressed as pg/ml. The interquartile range is shown in parentheses

[h] P-values were adjusted for demographic and clinical variables as well as inflation of significance described in the Methods.

Table 6

SNPs showing significant association with secreted IFNγ in the African-American cohort

SNP ID[a]	Chromosome[b]	Gene[c]	Location[d]	Distance from Gene	MAF[e]	Genotype	N[f]	Median (IQR)[g]	p-value[h]
rs7094333*	10	HPSE2	intron	0	11	AA	186	295.1 (20.2,1291.8)	1.04E-08
						AG	9	−27.6 (−171.5,21.3)	
						GG	1	−652.8 (−652.8,−652.8)	
rs1437635	11	SOX6	intron	0	11	CC	185	245.7 (0,1153)	2.51E-08
						CA	11	516.4 (277.4,4058.7)	
						AA	0	(,)	
rs3179690	1	UBXD3	3′UTR	0	15	GG	181	289.9 (20,1298.7)	3.11E-08
						GA	15	−31.2 (−160.4,728)	
						AA	0	(,)	
rs1453654	11	OR10G6	3′downstream	557	12	GG	185	289.9 (19.1,1298.7)	1.41E-07
						GA	10	20.4 (−218.6,304.5)	
						AA	1	−652.8 (−652.8,−652.8)	
rs11216816	11	AMICA1	intron	0	63	CC	138	361.4 (34.3,1321)	4.50E-07
						CA	53	131.2 (−124.7,1059.1)	
						AA	5	12.1 (−149.4,190.1)	
		LOC100128245	5′upstream	5799	63	CC	138	361.4 (34.3,1321)	4.50E-07
						CA	53	131.2 (−124.7,1059.1)	
						AA	5	12.1 (−149.4,190.1)	

[a] rs SNP identification number

[b] Chromosomal location of the indicated SNP

[c] Gene or genetic region containing the indicated SNP

[d] Location of the SNP relative to the gene

[e] Minor Allele Frequency

[f] Number of subjects with a given genotype

[g] Median outcome measurement for each genotype group. Results expressed as pg/ml. The interquartile range is shown in parentheses

[h] P-values were adjusted for demographic and clinical variables as well as inflation of significance described in the Methods.

[*] Sensitivity analysis p-values for rs7094333 = 1.11E-05.