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RESEARCH ARTICLE

Fc Gamma Receptor 3A Polymorphism and Risk for HIV-Associated Cryptococcal Disease

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ABSTRACT *Cryptococcus neoformans* is one of the most common causes of fungal disease in HIV-infected persons, but not all of those who are infected develop cryptococcal disease (CD). Although CD4$^+$ T cell deficiency is a risk factor for HIV-associated CD, polymorphisms of phagocytic Fc gamma receptors (FCGRs) have been linked to CD risk in HIV-uninfected persons. To investigate associations between FCGR2A 131 H/R and FCGR3A 158 F/V polymorphisms and CD risk in HIV-infected persons, we performed PCR-based genotyping on banked samples from 164 men enrolled in the Multicenter AIDS Cohort Study (MACS): 55 who were HIV infected and developed CD and a matched control group of 54 who were HIV infected and 55 who were HIV uninfected. Using additive and allelic statistical models for analysis, the high-affinity FCGR3A 158V allele was significantly associated with CD status after adjusting for race/ethnicity (odds ratio [OR], 2.1; $P = 0.005$), as was the FCGR3A 158 VV homozygous genotype after adjusting for race/ethnicity, rate of CD4$^+$ T cell decline, and nadir CD4$^+$ T cell count (OR, 21; $P = 0.005$). No associations between CD and FCGR2A 131 H/R polymorphism were identified. In binding studies, human IgG (hIgG)-*C. neoformans* complexes exhibited more binding to CHO-K1 cells expressing FCGR3A 158V than to those expressing FCGR3A 158F, and in cytotoxicity assays, natural killer (NK) cells expressing FCGR3A 158V induced more *C. neoformans*-infected monocyte cytotoxicity than those expressing FCGR3A 158F. Together, these results show an association between the FCGR3A 158V allele and risk for HIV-associated CD and suggest that this polymorphism could promote *C. neoformans* pathogenesis via increased binding of *C. neoformans* immune complexes, resulting in increased phagocyte cargo and/or immune activation.

IMPORTANCE HIV-associated CD4$^+$ T cell deficiency is a sine qua non for HIV-associated cryptococcal disease (CD), but not all patients with CD4$^+$ T cell deficiency develop CD despite serological evidence of previous infection. At present, there are no biomarkers that predict HIV-associated CD risk. The goal of our study was to understand whether Fc gamma receptor (FCGR) polymorphisms that have been shown to portend CD risk in HIV-uninfected people are associated with CD risk in HIV-infected people. Such biomarkers could identify those who would benefit most from targeted prophylaxis and/or earlier treatment, particularly in sub-Saharan Africa, where there are nearly a million cases of HIV-associated CD annually. A biomarker of risk could also identify potential candidates for immunization, should there be a vaccine for *Cryptococcus neoformans*.

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*C*ryptococcus neoformans is the main cause of fungal meningitis in HIV-infected and HIV-uninfected individuals. Human infection with *C. neoformans*, which occurs after acquisition of the fungus from the environment by inhalation, results in a state of latency that is thought to follow colonization of the respiratory tree (1). Most cases of clinically manifest cryptococcal disease (CD) are due to a breakdown in latency, which is most commonly caused by the progressive loss of CD4$^+$ T cells that occurs in those with HIV infection (2, 3). However, given that not all HIV-infected individuals with CD4$^+$ T cell deficiency develop CD despite serological evidence of previous infection (4–7), additional markers are needed to predict CD risk. Such biomarkers could

have a major impact by identifying those who would benefit most from targeted prophylaxis and/or earlier treatment. HIV-associated CD remains a major cause of morbidity and mortality globally, particularly in sub-Saharan Africa, where nearly a million cases and half a million deaths due to CD are estimated to occur annually (8).

Although HIV infection is a strong risk factor for CD, CD also occurs in apparently immunocompetent individuals and patients with other types of immune impairment, including that caused by corticosteroids and immunosuppressive agents used for solid-organ transplantation (3, 9–11). To date, a unifying defect that explains CD risk in immunosuppressed as well as apparently im-

munocompetent patients has not been identified. IgM and memory B cells have been implicated in resistance to HIV-associated CD (12), and studies in mice have linked these factors to containment of *C. neoformans* in the lungs and prevention of dissemination to the brain (13, 14). However, it is clear that additional factors contribute to CD risk and, in this regard, antibody immunity has been the focus of many studies over the past decade and a half. Serum IgG reactive with the glucuronoxylomannan (GXM) component of cryptococcal capsular polysaccharide (GXM-IgG) and *C. neoformans* proteins has been identified in HIV-infected and HIV-uninfected adults and children (4–7, 15, 16), and many studies show that GXM-IgG enhances macrophage phagocytosis of *C. neoformans* (17–20).

IgG mediates phagocytosis via Fc gamma receptors (FCGR) (21), which were required for a mouse GXM-IgG1 monoclonal antibody to protect mice against lethal *C. neoformans* infection (22). On the other hand, human IgG1 enhanced CD in mice, while IgG2 and IgG4 were protective (23). Although this could have been owed in part to species differences in human IgG-mouse FCGR binding, human IgG2 mediates phagocytosis via (human) FCGR2A, the only FCGR to which it binds (24). Underscoring the role that IgG2-FCGR2A binding could play in protection against *C. neoformans*, immune sera from recipients of an experimental GXM-TT vaccine mediated human macrophage *C. neoformans* phagocytosis via FCGR2A (18). Thus, it is logical to posit that GXM-IgG could influence host defense against *C. neoformans* via IgG subclass binding to FCGRs and be affected by FCGR polymorphism. Indeed, allelic polymorphisms of phagocytic FCGRs, namely, FCGR2A 131 H/R (rs1801274) and FCGR3A 158 F/V (rs396991), were associated with CD risk in HIV-uninfected Caucasians (25), whereas 232I/T (rs1050501) polymorphism of the nonphagocytic, inhibitory FCGR2B, but not FCGR3A 158 F/V, was associated with CD risk in a Chinese cohort (26).

In this study, we sought to extend the aforementioned associations between FCGR2A (131H/R) and FCGR3A (158F/V) genetic polymorphisms and CD risk in HIV-uninfected individuals to HIV-infected individuals enrolled in the Multicenter AIDS Cohort Study (MACS). We chose to focus on polymorphisms of these low-affinity phagocytic receptors because their polymorphic variants bind IgG with different affinities and thus influence FCGR effector functions, including phagocytosis and antibody-dependent cytotoxicity (ADCC). The FCGR2A 131-H allele binds IgG2 with higher affinity than the R allele (24, 27) and is effectively the only FCGR that binds human IgG2. The FCGR3A 158V allele binds all IgG subclasses with higher affinity than the 158F allele (24, 27, 28). Our data revealed an association between the FCGR3A 158V allele and HIV-associated CD after adjusting for demographic and clinical characteristics and factors also associated with CD risk, e.g., the rate of CD4+ T cell decline and nadir CD4+ T cell count.

RESULTS

Study population characteristics. The study population consisted of 164 homosexual/bisexual men enrolled in the Multicenter AIDS Cohort Study (MACS), an ongoing study of the natural history of HIV infection (29). Banked serum samples from (i) 55 HIV-infected participants who developed a confirmed (culture- and/or antigen-positive) case of CD (HIV-positive [HIV+] CD+ group); (ii) 54 HIV-infected participants who did not develop CD matched to HIV+ CD+ participants on age, re-

cruitment site, and CD4+ T cell levels at enrollment (HIV+ CD− group); and (iii) 55 HIV-uninfected participants with no history of CD matched to the 55 HIV+ CD+ subjects by age and recruitment site with no history of CD (HIV− CD− group) were used to obtain DNA for genotyping and serological studies. The matching criteria for this nested case-control study are described in detail in Materials and Methods.

Demographic and clinical characteristics of the study populations are shown in Table 1. All participants were male, the majority (81%) were non-Hispanic white, and the median age was 34 years (interquartile range [IQR], 30 to 40 years). As expected given the matched case-control study design, the groups did not differ by age or, among HIV-positive participants, by CD4+ T cell level at the first HIV-positive study visit (medians of 605 and 619 cells/μl for the HIV+ CD+ and HIV+ CD− groups, respectively; $P = 0.80$). The HIV+ CD+ group did, however, have a shorter time to first AIDS diagnosis and lower nadir CD4+ T cell level and initiated highly active antiretroviral therapy (HAART) earlier than the HIV+ CD− group (Table 1), suggesting that the rate of HIV disease progression after matching was higher for the HIV+ CD+ than for the HIV+ CD− group.

Associations between FCGR3A and FCGR2A genotypes and CD. Genotyping for FCGR2A 131 H/R and FCGR3A 158 F/V polymorphisms was performed using gene-specific primers and PCR amplification followed by sequencing, as described in Materials and Methods. The genotype frequencies of FCGR2A 131 H/R (HH, HR, and RR) and FCGR3A 158 F/V (FF, FV, and VV) in the HIV+ CD+, HIV+ CD−, and HIV− CD− groups are shown in Table 2. Notably, the high-affinity FCGR3A 158 VV genotype was more common among men who developed CD (22%) than among the controls (9% in the combined HIV+ CD− and HIV− CD− groups).

In multivariable analyses based on different genetic models of inheritance (additive, dominant, and recessive), we observed consistent significant associations between the FCGR3A 158V allele and risk of CD, with odds ratios (ORs) ranging between 2.1 and 3.1 (Table 3). In an allelic model that allowed us to examine FCGR3A 158 VV homozygosity and 158 FV heterozygosity separately, there was a strong significant association between CD status and the FCGR3A 158 VV genotype (OR, 4.5; 95% confidence interval [CI], 1.5 to 13.1; $P = 0.007$). CD risk was also elevated for men with the FCGR3A 158 FV heterozygous genotype, but this association was not statistically significant (OR, 2.0; 95% CI, 0.9 to 4.4; $P = 0.08$). When analyses were restricted to non-Hispanic white men or to HIV-infected participants alone, the association between the FCGR3A 158 VV genotype and CD risk was significant among non-Hispanic whites (OR, 3.6; 95% CI, 1.2 to 11.0; $P = 0.03$) but that for HIV-infected participants was not statistically significant (OR, 2.5; 95% CI, 0.8 to 8.3; $P = 0.14$), most likely because of smaller sample sizes and reduced statistical power (Table 3). No significant associations were observed between FCGR2A 131 H/R polymorphism and CD in any of the statistical models (Table 3).

Adjustment for rate of CD4+ decline and nadir in analyses of FCGR3A and CD. Since the rates of HIV disease progression differed between the HIV+ CD+ and HIV+ CD− groups following their matching visits (Table 1), we controlled for both the rate of CD4+ decline and the CD4+ nadir to minimize the possibility that the observed association between FCGR3A 158 F/V polymorphism and CD might reflect a link between FCGR3A and HIV

TABLE 1 Characteristics of the study population[a]

Study participant characteristic	Value(s) for indicated population ($n = 164$)		
	HIV$^+$ CD$^+$ ($n = 55$)	HIV$^+$ CD$^-$ ($n = 54$)	HIV$^-$ CD$^-$ ($n = 55$)
Median (IQR) age	34 (29–37)	34 (30–37)	35 (31–45)
No. with race/ethnicity:			
White non-Hispanic	43	48	42
Other[b]	12	6	13
No. with yr of first HIV-positive study visit:			
1984–1986	45	44	NA
1987–1990	10	10	NA
No. with CD4$^+$ count at first HIV-positive visit (cells/μl):			
<200	1	2	NA
200–500	16	13	NA
>500	38	39	NA
Median (IQR) CD4$^+$ nadir (cells/μl)[c]	42 (16–133)	75 (16–295)	NA
No. with CD4$^+$ nadir (cells/μl):			
<50	29	22	NA
50–99	9	6	NA
100–199	8	9	NA
≥200	9	17	NA
No. with yr of first HAART[c]:			
1993–1996	54	42	NA
1997–2000	1	11	NA
2001–2005	0	1	NA
No. with clinical AIDS[c,d]	55	32	NA
Median (IQR) no. of yrs from first HIV-positive visit to clinical AIDS[c,e]	5.8 (3.8–7.4)	8.3 (6.5–9.5)	NA

[a] HAART, highly active antiretroviral therapy; IQR, interquartile range; NA, not applicable.
[b] Other races and ethnicities included the following: for the HIV$^+$ CD$^+$ population, Hispanic ($n = 5$), black non-Hispanic ($n = 4$), Asian or Pacific Islander ($n = 1$), and other race/ethnicity ($n = 2$); for the HIV$^+$ CD$^-$ population, Hispanic ($n = 1$) and black non-Hispanic ($n = 5$); and for the HIV$^-$ CD$^-$ population, Hispanic ($n = 5$), black non-Hispanic ($n = 7$), and other race/ethnicity ($n = 1$).
[c] Significantly different ($P < 0.05$) by a t test or Fisher's exact test.
[d] In the HIV$^+$ CD$^-$ control group, the most common AIDS diagnoses were *Pneumocystis jirovecii* pneumonia, Kaposi's sarcoma, cytomegalovirus infection, atypical mycobacterial infection, and *Candida* esophagitis.
[e] Among study participants who received a clinical AIDS diagnosis.

disease progression, an association observed in a prior study (30). Under an additive model of genetic inheritance with adjustments for CD4$^+$ T cell nadir and decline in addition to race/ethnicity, we observed a significant association between the FCGR3A 158V allele and CD (OR, 3.3; 95% CI, 1.4 to 7.9; $P = 0.007$). The magnitude of this association was similar under a dominant model of genetic inheritance, although not statistically significant (Table 4). However, significant associations were observed under a recessive model (OR, 17.6; 95% CI, 2.1 to 148.2; $P = 0.008$) and in the allelic model, where the FCGR3A 158 VV genotype had a significant association with CD (OR, 20.9; 95% CI, 2.5 to 177.3; $P = 0.005$)

but the FCGR3A 158 FV genotype did not (OR, 1.7; 95% CI, 0.5 to 5.5; $P = 0.41$). A rapid CD4$^+$ T cell decline following matching, as defined in Materials and Methods, was also a significant, independent risk factor for CD under each model of genetic inheritance (Table 4). A CD4$^+$ nadir of <50 cells/μl and other race/ethnicity were also associated with CD, with significant or borderline significant associations under each model of genetic inheritance.

Total serum IgG1, IgG2, and IgG3 levels and antibodies to GXM. Next, we investigated the relationship between total and GXM-IgG levels and CD status. No associations between serum IgG subclasses or GXM-IgG levels and CD were observed. There

TABLE 2 Fc gamma receptor genotype frequencies in the study population

Study population	No. (and/or %) of participants with each genotype frequency					
	FCGR3A 158 F/V			FCGR2A 131 H/R		
	FF	FV	VV	HH	HR	RR
All participants ($n = 164$)	60 (36)	82 (50)	22 (13)	22 (13)	97 (59)	45 (27)
HIV$^+$ CD$^+$ ($n = 55$)	13 (23)	30 (54)	12 (22)	8 (14)	32 (58)	15 (27)
HIV$^+$ CD$^-$ ($n = 54$)	24 (44)	27 (50)	3 (5)	5 (9)	34 (63)	15 (27)
HIV$^-$ CD$^-$ ($n = 55$)	23 (42)	25 (45)	7 (12)	9 (16)	31 (56)	15 (27)
Caucasians[a]	(50)	(39)	(11)	(31)	(44)	(25)
African Americans[b]	(42)	(50)	(8)	(26)	(43)	(31)

[a] Genotype frequencies in normal healthy U.S. Caucasians (44).
[b] Genotype frequencies in normal healthy U.S. African Americans (44).

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TABLE 3 Associations between FCGR3A and FCGR2A polymorphisms and CD by genetic model of inheritance[a]

Study population	FCGR3A 158 F/V			FCGR2A 131 H/R		
	Inheritance	OR (95% CI)	P	Inheritance	OR (95% CI)	P
All men ($HIV^+ CD^+$ versus $HIV^+ CD^-$ and $HIV^+ CD^-$; $n = 164$)	Additive	2.1 (1.2–3.5)	0.005	Additive	1.0 (0.7–1.3)	0.79
	Dominant	2.4 (1.2–5.1)	0.02	Dominant	0.9 (0.3–2.1)	0.74
	Recessive	3.1 (1.2–8.0)	0.02	Recessive	1.0 (0.5–2.0)	0.97
	Allelic			Allelic		
	FF	1.0		HH	1.0	
	FV	2.0 (0.9–4.4)	0.08	HR	0.9 (0.3–2.2)	0.74
	VV	4.5 (1.5–13.1)	0.007	RR	0.9 (0.3–2.4)	0.79
Non-Hispanic white men ($HIV^+ CD^+$ versus $HIV^+ CD^-$ and $HIV^+ CD^-$; $n = 133$)	Additive	1.9 (1.1–3.3)	0.02	Additive	0.8 (0.5–1.5)	0.51
	Dominant	2.3 (1.0–5.4)	0.05	Dominant	0.8 (0.3–2.3)	0.73
	Recessive	2.5 (0.9–6.5)	0.07	Recessive	0.8 (0.3–1.8)	0.53
	Allelic			Allelic		
	FF	1.0		HH	1.0	
	FV	1.9 (0.8–4.8)	0.17	HR	0.9 (0.3–2.5)	0.82
	VV	3.6 (1.2–11.0)	0.03	RR	0.7 (0.2–2.3)	0.54
HIV-positive men ($HIV^+ CD^+$ versus $HIV^+ CD^-$; $n = 109$)	Additive	1.7 (1.0–3.0)	0.08	Additive	1.0 (0.5–1.8)	0.93
	Dominant	2.1 (0.9–4.6)	0.07	Dominant	1.0 (0.4–2.8)	0.93
	Recessive	1.8 (0.6–5.4)	0.30	Recessive	0.9 (0.4–2.3)	0.84
	Allelic			Allelic		
	FF	1.0		HH	1.0	
	FV	2.0 (0.8–4.6)	0.12	HR	1.1 (0.4–3.0)	0.89
	VV	2.5 (0.8–8.3)	0.14	RR	1.0 (0.3–3.2)	0.95

[a] Data were adjusted for race/ethnicity, age, recruitment site, recruitment wave, $CD4^+$ T-cell level (± 50 at the first HIV-positive study visit), availability of serum samples ± 2 years apart, and duration of CD-free follow-up time.

was also no significant difference in total serum IgG1, IgG2, IgG3, or GXM-IgG levels between the $HIV^+ CD^+$ and $HIV^+ CD^-$ groups (Fig. 1). However, each HIV-infected ($HIV^+ CD^+$ and $HIV^+ CD^-$) group had more serum IgG1 and IgG3 and less IgG2 than the HIV-uninfected ($HIV^- CD^-$) group (Fig. 1A to C). The levels of GXM-IgG did not differ between the HIV-infected

groups, but each group ($HIV^+ CD^+$ and $HIV^+ CD^-$) had a significantly higher level than the HIV-uninfected group (Fig. 1D).

IgG-*C. neoformans* immune complexes have a higher frequency of binding to CHO-K1 cells expressing FCGR3A 158V than to those expressing FCGR3A 158F. Since FCGR3A 158V was associated with CD risk in our HIV-infected cohort and in a

TABLE 4 Associations between FCGR3A 158 F/V polymorphisms and CD in HIV-positive men, controlling for race/ethnicity, rate of $CD4^+$ T cell decline and $CD4^+$ nadir, by model of genetic inheritance

Parameter	No. of men ($n = 109$)	Value(s) for indicated inheritance model[a]							
		Additive		Dominant		Recessive		Allelic	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
FCGR3A 158 F/V genotype		3.3 (1.4–7.9)	0.007	2.9 (1.0–8.4)	0.06	17.6 (2.1–148.2)	0.008		
FF	37	—[b]		—		—		1.0	
FV	57	—		—		—		1.7 (0.5–5.5)	0.41
VV	15	—		—		—		20.9 (2.5–177.3)	0.005
$CD4^+$ status[c]									
Stable $CD4^+$ count	34	1.0		1.0		1.0		1.0	
Heterogeneous $CD4^+$ decline	62	1.4 (0.4–4.9)	0.56	1.8 (0.6–5.7)	0.31	1.4 (0.4–5.2)	0.60	1.4 (0.4–5.0)	0.64
Rapid $CD4^+$ decline	13	31.6 (2.3–438.2)	0.01	24.4 (2.3–263.6)	0.008	48.6 (2.9–807.3)	0.007	43.4 (2.6–736.5)	0.009
$CD4^+$ nadir									
>200	26	1.0		1.0		1.0		1.0	
100–200	17	1.3 (0.2–8.3)	0.78	1.1 (0.2–6.4)	0.89	0.9 (0.1–6.7)	0.94	1.1 (0.2–8.2)	0.93
50–100	15	5.3 (0.8–34.1)	0.08	5.0 (0.8–30.1)	0.08	4.0 (0.7–23.4)	0.12	4.5 (0.7–28.8)	0.11
<50	51	6.1 (1.0–35.0)	0.04	4.2 (0.9–19.7)	0.07	5.8 (0.9–37.9)	0.07	6.4 (1.0–41.6)	0.05
Race/ethnicity									
White non-Hispanic	91	1.0		1.0		1.0		1.0	
Other race/ethnicity	18	9.0 (1.2–65.5)	0.03	6.2 (1.0–37.7)	0.05	15.6 (1.8–133.3)	0.01	14.0 (1.6–124.1)	0.02

[a] Results are from a single multivariable conditional logistic regression model that included all covariates shown in the table.
[b] Only a single odds ratio for FCGR3A 158 F/V is produced in additive, dominant, and recessive models.
[c] $CD4^+$ statuses are defined in Materials and Methods.
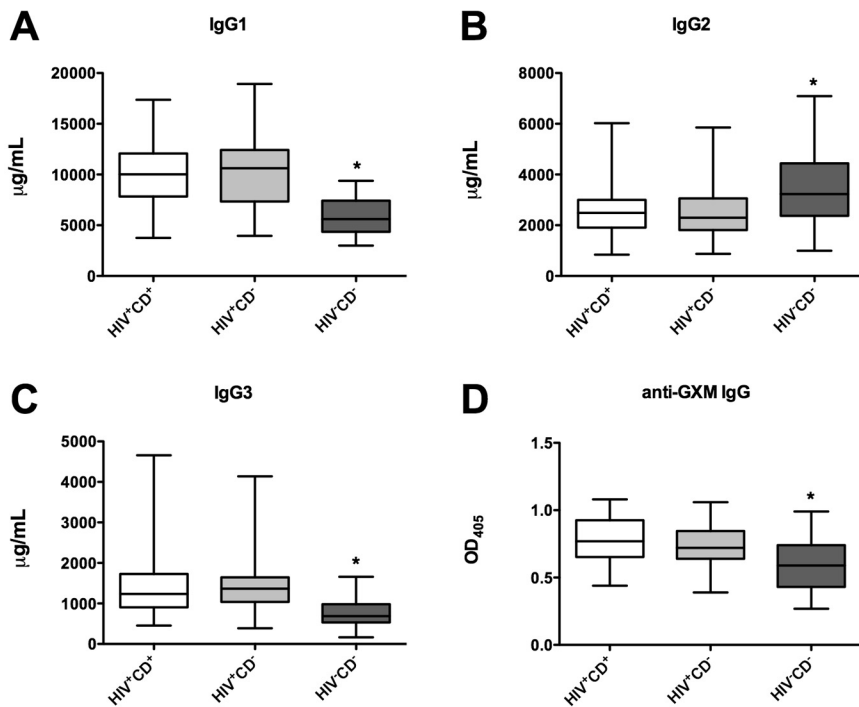
FIG 1 Total immunoglobulin levels in 164 human serum samples obtained from MACS. (A to C) Serum IgG1 (A), IgG2 (B), and IgG3 (C) levels were determined using radial immunodiffusion kits as described in the text. The y axis indicates concentrations in μg/ml for the groups depicted on the x axis. (D) Absorbance values shown for serum GXM-IgG levels. The white boxes represent the HIV$^+$ CD$^+$ group, the gray boxes represent the HIV$^+$ CD$^-$ group, and the black boxes represent the HIV$^-$CD$^-$ group. Lines across each box show the median for each group, boundaries of the box represent the 25th and 75th percentiles, and lines extending from each box represent the 1st and 99th percentile. P values to compare medians between groups were calculated using the Mann-Whitney test. Asterisks (*), significant differences between the HIV-infected (HIV$^+$ CD$^+$ and HIV$^+$ CD$^-$) and HIV-uninfected (HIV$^-$ CD$^-$) groups; OD$_{405}$, optical density at 405 nm.

previous study of HIV-uninfected individuals (25), we compared the affinities of FCGR3A 158V and FCGR3A 158F for IgG-opsonized *C. neoformans* using CHO-K1 cells transfected with these receptors. For these studies, surface expression of FCGR3A was first confirmed using MEM-154, a CD16 monoclonal antibody that was found to have higher affinity for the 158V allotype (31, 32). As expected, the fluorescence intensities of CHO-K1 cells expressing the FCGR3A 158V allotype were higher than the intensities seen with those expressing the 158F allotype (Fig. 2A). Subsequently, immune complexes created with green fluorescent protein (GFP)-expressing *C. neoformans* and heat-inactivated normal human serum (serum opsonized) or individual human monomeric IgGs—IgG1, IgG2, IgG3, and IgG4 (IgG opsonized)—were incubated with allophycocyanin (APC)-labeled CHO-K1 cell lines (expressing 158F or 158V receptors) and binding was detected by scoring GFP$^+$ APC$^+$ cells by flow cytometry (Fig. 2B and C). Staining controls are shown in Fig. S1 in the supplemental material. FCGR3A 158V-expressing CHO-K1 cells bound serum-opsonized *C. neoformans* (9.9%) with a higher frequency than 158F-expressing cells (7.4%; $P = 0.04$; Student's t test) (Fig. 2D). *C. neoformans* opsonized with individual monomeric human IgG1, IgG2, IgG3, or IgG4 (in concentrations ranging from 1,000 μg/ml to 0.01 μg/ml) or a combination using each IgG subclass also exhibited a trend toward a higher frequency of binding to FCGR3A 158V-expressing than to FCGR3A 158F-

expressing CHO-K1 cells, but this did not reach statistical significance ($P < 0.1$) (Fig. 2E).

Anticryptococcal activity of NK cells is FCGR3A independent. Natural killer (NK) cells are a major cell type expressing FCGR3A. Thus, the high-affinity 158V polymorphism might affect the antifungal activity of NK cells against *C. neoformans* in an antibody-dependent fashion via antibody-dependent cellular cytotoxicity (ADCC) (33) or in an antibody-independent, direct (34) fashion. To examine the effect of NK cell FCGR3A 158 F/V polymorphism on antifungal activity, we incubated NK cell lines expressing (i) FCGR3A 158F or (ii) FCGR3A 158V and (iii) cells lacking an FCGR3A receptor (parental cell line) with *C. neoformans* strains 24067 and Cap67 (acapsular mutant) as described in Materials and Methods. All three NK cell lines mediated significant antifungal activity after 24 h of incubation (Fig. 3A), which increased after 48 h (Fig. 3B). However, there were no differences in fungal killing as a function of FCGR3A 158V or 158F expression. Hence, our data suggest that NK cell antifungal activity is not affected by FCGR3A 158 F/V polymorphism, with the caveat that our results reflect the activity of an activated NK cell tumor line.

FCGR3A 158V polymorphism mediates more NK cell-dependent ADCC. As the direct, antibody-independent antifungal activity of NK cells was independent of FCGR3A 158 F/V polymorphism, we examined the relationship between antibody-dependent NK-mediated ADCC and FCGR3A 158 F/V polymorphism. For these experiments, we used the NK cell lines expressing FCGR3A 158V or 158F as effector cells and *C. neoformans*-infected human CD14$^+$ CD16$^+$ monocytes (prepared as described in Materials and Methods) as target cells. ADCC was tested in the presence of heat-inactivated normal human serum (10 μg/ml) or total IgG (10 μg/ml) (Fig. 3C). In the presence of total IgG, FCGR3A 158V-expressing NK cells induced more cytotoxicity (80.5%) than cells expressing 158F (23%; $P = 0.01$) or those lacking FCGR3A (19.3%; $P = 0.01$). Similarly, in the presence of normal human serum, FCGR3A 158V-expressing NK cells induced more cytotoxicity (72.4%) than those expressing 158F (28%; $P = 0.01$) or those lacking FCGR3A (21.3%; $P = 0.006$). The levels of cytotoxicity were similar for all three NK cell lines (158F, 158V, and parental) in the absence of human serum or total IgG. Furthermore, while target cell CFU were decreased in the presence of human serum or total IgG compared to no-antibody controls, there were no differences in target cell CFU for any of the NK cell lines with either antibody source (data not shown). Hence, our findings suggest that NK FCGR3A 158 F/V polymorphism could affect *C. neoformans* pathogenesis in an antibody-dependent fashion via ADCC, again with the caveat that our data reflect the activity of an activated NK tumor cell line.
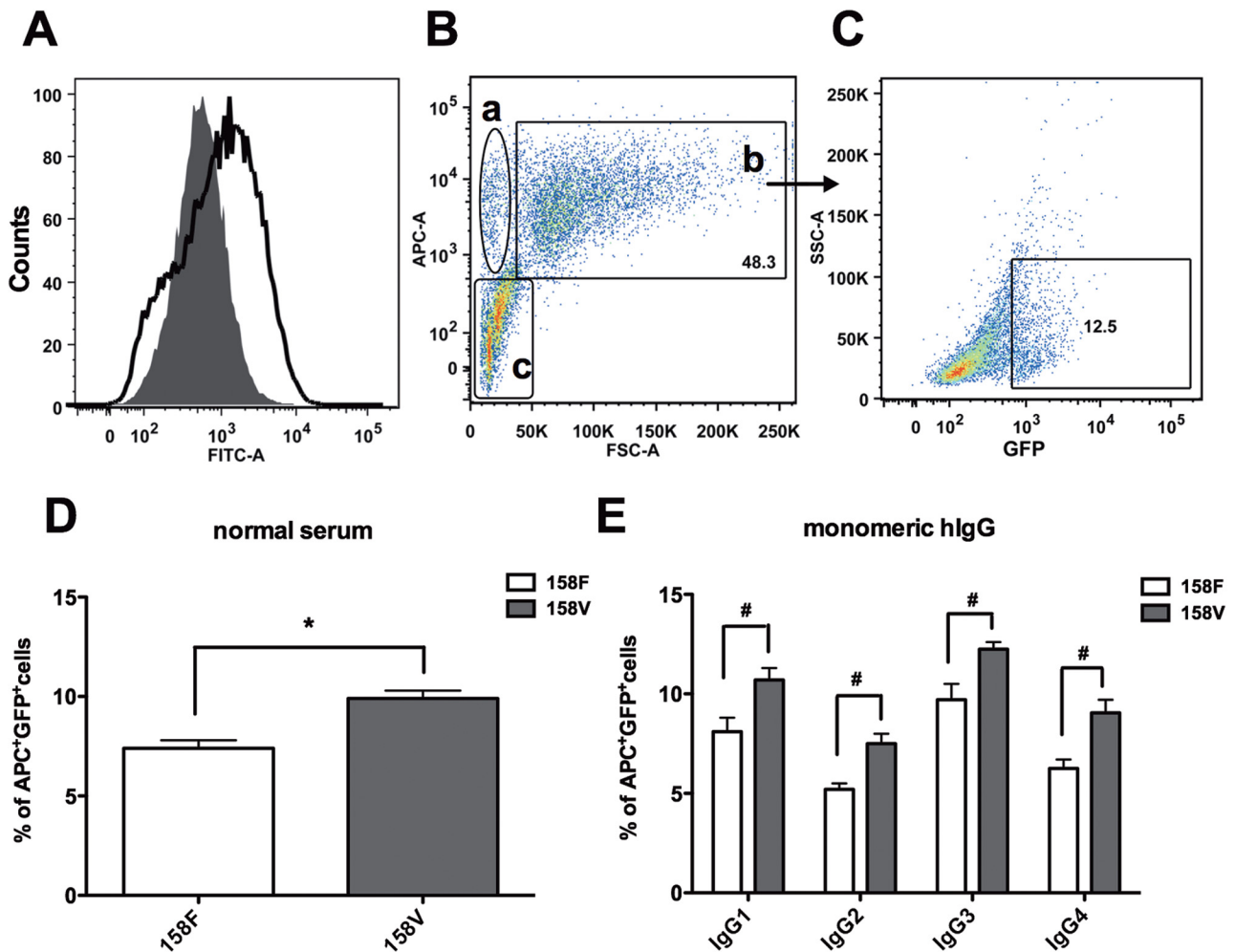
FIG 2 Binding of human Ig-*C. neoformans* immune complexes to CHO-K1 cell lines expressing FCGR3A 158F or 158V receptors. (A) Flow cytometric analysis of CHO cell lines expressing 158F (shaded plot) and 158V (dark line) receptors stained with CD16 MEM-154 antibody followed by FITC-conjugated goat anti-mouse IgG antibody. The histograms show the data for 50,000 events from each sample. (B) CHO cells were stained with APC-labeled antibodies recognizing fibronectin receptor and incubated with immune complexes comprising GFP-expressing *C. neoformans* and human immunoglobulins. The boxed insert (gate b) indicates a population that contains APC$^+$ CHO cells. Free *C. neoformans* cells (gate c) and dead cells (gate a) were excluded. (C) APC$^+$ CHO cells were further gated on the GFP channel to detect binding to precomplexed human Ig and GFP-expressing *C. neoformans*. The boxed insert indicates a population that contains CHO cells that bind immune complexes. Plots shown are representative of two independent experiments. (D) The proportions of CHO cells (expressing 158F or 158V receptors) binding to immune complexes comprising total normal human serum (0.1 µg/ml) and GFP-*C. neoformans* (10^5) are shown as percentages. (E) CHO-K1 cells (expressing 158F or 158V) binding to immune complexes comprising human monomeric IgG subclasses (0.1 µg/ml) and GFP-*C. neoformans* (10^5) are depicted as percentages for IgG1, IgG2, IgG3, and IgG4. An asterisk (*) indicates significant differences between groups ($P < 0.05$); octothorpes (#) indicate nearly significant differences between groups ($P < 0.1$) (Student's *t* test).

DISCUSSION

Our data demonstrate a strong and significant association between the high-affinity FCGR3A 158V allele and CD risk in HIV-infected individuals. This confirms and extends the results of a previous study by Meletiadis et al., which identified the same association in HIV-uninfected individuals (25). On the other hand, Hu et al. did not find an association between FCGR3A 158V polymorphism and CD in a Chinese cohort (26). Thus, this association appears to be strongest for Caucasians, with the caveat that together, the cohorts in these studies were small and genetically diverse with different comorbid conditions that could affect risk. However, despite finding an association between FCGR3A 158V polymorphism and CD risk, we did not find an association between CD risk and the low-affinity FCGR2A 131R allele, as reported by Meletiadis et al. (25). Our data suggest an explanation for this difference. FCGR2A is unique in its capacity to interact with human IgG2 (24) and was previously shown to be responsible for immune serum-mediated phagocytosis of *C. neoformans* by human peripheral blood mononuclear cells (PBMCs) (18). IgG2 is the predominant subclass of human GXM-IgG (5, 35) and, as reported previously for patients with HIV/AIDS (4, 5, 7), HIV-infected participants in our study had less IgG2 than HIV-uninfected participants. Thus, together with the study by Meletiadis et al. (25), our data suggest that HIV status does not affect the association between the FCGR3A 158V allele and CD risk, whereas the risk conferred by FCGR2A 131R could be limited to HIV-uninfected individuals. Given that HIV infection is marked by IgG2 deficiency, it is logical to posit that an inability of
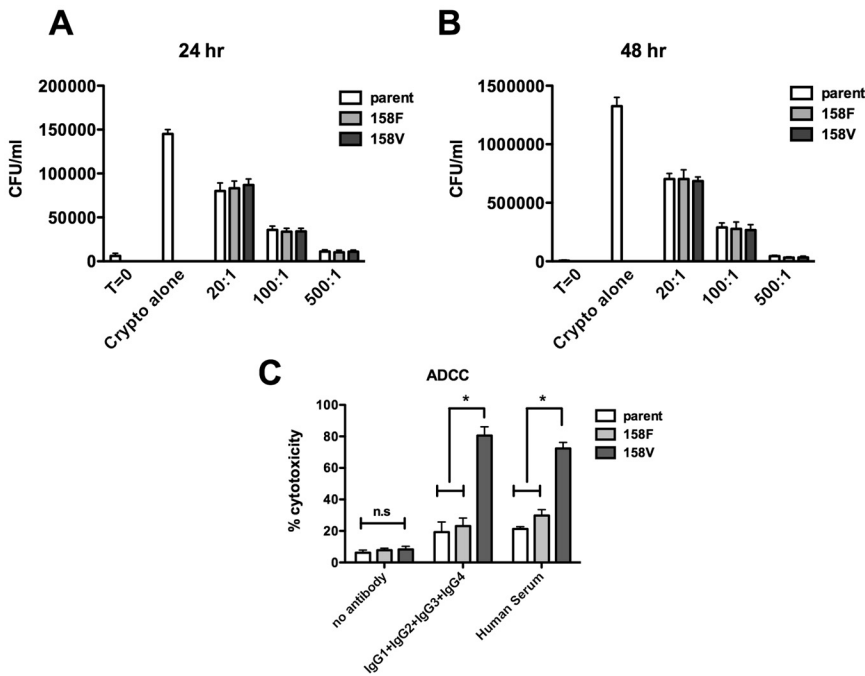
FIG 3 Assessing anticryptococcal activity of NK cells. (A and B) NK cell lines expressing FCGR3A 158F (gray bars), FCGR3A 158V (black bars), or no FCGR3A (parental) (open bars) were incubated with *C. neoformans* strain 24067 for 24 h (A) and 48 h (B) at different effector/target (E/T) ratios as indicated on x axis. *C. neoformans* viability was analyzed by determining the number of CFU/ml as indicated on y axis. The numbers of *C. neoformans* cells at the start of the assay and at the indicated end times were included to account for the 10-to-500-fold growth of the organisms during the course of the assay. T = 0, zero time; Crypto alone, incubation in the absence of NK cells. Similar results were obtained using acapsular *C. neoformans* strain CAP67 (not shown). (C) Cytotoxicity assay performed by measuring LDH release. FCGR3A polymorphic NK cells (1×10^5/well) were added to a 96-well round-bottom plate and coincubated with target cells (*C. neoformans*-infected CD14$^+$ CD16$^+$ monocytes) (1×10^4/well) at 37°C and 5% CO$_2$ for 4 h in the presence of normal human serum (10 μg/ml) and pooled IgG (10 μg/ml) as indicated on the x axis. Percent cytotoxicity was calculated based on total content and spontaneous release of LDH. Values are shown as means ± standard errors of the means of the results from two different experiments. Asterisks (*), significant differences between groups ($P < 0.05$); n.s., not significant (Student's t test).

this hypothesis, CD14$^+$ CD16$^+$ monocytes are expanded in HIV-infected individuals (41) and one way in which *C. neoformans* has been proposed to disseminate to the brain is within monocytes via a Trojan horse mechanism (42). Although studies with GXM-IgG and CD14$^+$ CD16$^+$ monocytes with known FCGR3A 158 F/V expression were beyond the scope of this study, our data provide a potential mechanistic explanation for how the FCGR3A 158V allele could enhance CD risk via monocyte-borne *C. neoformans* dissemination to the brain. The high-affinity FCGR3A 158V allele could also promote increased *C. neoformans* binding/uptake by macrophages, with the caveats that FCGR3A binding does not always trigger microbial uptake (43) and that immune activation rather than antibody-mediated enhancement of infection has been implicated in the association between FCGR3A 158V allele and HIV infection and progression (30).

Our results suggest that NK cell-mediated ADCC could also enhance *C. neoformans* virulence. The risk for Kaposi's sarcoma (KS) in HIV-infected individuals was linked to NK cell-mediated ADCC via FCGR3A 158V polymorphism (44, 45), with the caveat that these studies predated the use of antiretroviral therapy and KS could have been a surrogate for HIV progression. Nonetheless, NK cell FCGR3A is a critical effector of ADCC and lysis of infected, inflammatory, and malignant cells. Regarding the latter, FCGR3A 158V was implicated in the rituximab response of patients with non-Hodgkin's lymphoma (46). Our ADCC experiments show that in the presence of normal human serum or IgG, NK cells expressing 158V mediated more cytotoxicity of *C. neoformans*-infected CD14$^+$ CD16$^+$ human monocytes than cells expressing 158F. On the other hand, in the absence of an antibody source, NK cell-mediated cytotoxicity and antifungal activity were independent of FCGR3A genotype. However, we note that the activation state of the NK cell tumor line used in our studies (47) could have influenced our results. As such, more studies are needed to translate the results from this study to natural NK cell effector function.

NK cells from healthy donors homozygous for the FCGR3A 158 VV genotype bind more IgG1, IgG3, and IgG4 than cells from 158 FF donors (32). Thus, it is reasonable to hypothesize that NK cell-mediated ADCC of *C. neoformans*-infected monocytes is a function of IgG subclass distribution and FCGR3A genotype and that higher levels of nonspecific IgG1, IgG3, and GXM-IgG in HIV-infected individuals could promote increased ADCC, monocyte/macrophage *C. neoformans* uptake, and/or immune activation in those with FCGR3A 158V. We did not examine immune activation in this study, but as noted above, CD14$^+$ CD16$^+$ monocytes secrete chemokines that damage the BBB and induce

IgG2 to mediate phagocytosis via the higher-affinity FCGR2A 131-H allele is detrimental to host defense in HIV-uninfected individuals but irrelevant in HIV-infected individuals due to the loss of IgG2.

Our data show that *C. neoformans*-hIgG complexes bound CHO-K1 cells expressing the FCGR3A 158V allotype with higher affinity than those expressing 158F. Current knowledge about *C. neoformans* virulence suggests that higher-affinity binding of immune complexes to FCGR3A 158V could increase monocyte/macrophage *C. neoformans* attachment and/or uptake (36–38), which could in turn increase intracellular cargo and replication. Along these lines, higher levels of nonspecific IgG1, IgG3, and GXM-IgG in HIV-infected individuals, as reported here, could form more *C. neoformans*-hIgG complexes, particularly as the fungal burden rises with progressive CD4$^+$ T cell deficiency. Of relevance to HIV-associated CD, CD14$^+$ CD16$^+$ monocytes (CD16: FCGR3A) produce chemokines that damage the blood-brain barrier (BBB) and have been implicated in central nervous system (CNS) inflammation and invasion of the CNS by HIV (39, 40). Thus, high-affinity *C. neoformans*-hIgG binding to CD14$^+$ CD16$^+$ monocytes via FCGR3A 158V could increase the likelihood of *C. neoformans* dissemination to the brain. In support of

brain inflammation (48) and immune activation was implicated in associations between FCGR3A 158V, HIV infection, and HIV progression (30). While beyond the scope of the current study, work is ongoing in our laboratory to determine the effect of FCGR3A polymorphisms on monocyte/macrophage activation and uptake and intracellular replication of *C. neoformans*.

Our data show that the rate of HIV progression was faster in the HIV$^+$ CD$^+$ group than in the HIV$^+$ CD$^-$ group following their matching visits. We accounted for these between-study group differences in light of recent reports of associations between FCGR3A 158V, HIV infection, and HIV disease progression (30) and between the FCGR3A 158 VV genotype and acquisition of HIV in low-risk recipients of an investigational gp120 vaccine (49). In our study, multivariable regression analysis revealed significant independent associations between FCGR3A 158V polymorphism, CD4$^+$ T cell nadir, and the rate of CD4$^+$ T cell decline and CD risk. While our analysis cannot fully exclude the possibility that FCGR3A 158V polymorphism was associated with HIV progression rather than CD in our cohort, to our knowledge, neither the rate of CD4$^+$ T cell decline nor other markers of the rate of HIV progression have been previously linked to CD.

Race/ethnicity other than non-Hispanic white was also associated with CD in our study. This is consistent with data from prior studies which also found that CD risk differed by race/ethnicity (50, 51). However, given that only 12 of the 55 HIV$^+$ CD$^+$ participants in our study were not non-Hispanic white, the study was not powered to examine relationships between races/ethnicities. As such, our data do not provide additional insight into the effect of race/ethnicity on CD risk. Given the strong association between FCGR3A genotype and risk of CD observed in the current investigation, it is possible that differences in the prevalence of FCGR3A genotypes across racial/ethnic groups might have contributed to associations between race/ethnicity and CD observed in prior studies. However, because one study showed that the prevalence of the FCGR3A 158 VV genotype (8%) in African Americans did not differ significantly from that in Caucasians (11%) (44), it is more likely that other genetic and/or epigenetic factors and comorbidities account for this association. This will be addressed in future studies. Nonetheless, our report clearly establishes a relationship between FCGR3A 158V polymorphism and HIV-associated CD, while having some important limitations that will be addressed in further studies. First, as our study was limited to males, the relationship between FCGR3A polymorphism and CD risk must be studied in females. Second, since this study was not stratified by CD manifestation, conclusions about the relationship between FCGR3A polymorphism and different clinical presentations of CD cannot be drawn. Also, we did not examine the FCGR2B polymorphism that was associated with CD risk in HIV-uninfected Chinese patients (26). Given that GXM binds this receptor and induces an inhibitory signal to macrophages (52), it is possible that differing affinities of GXM-IgG for FCGR2B could modify the activity of the phagocytic receptors FCGR2A and FCGR3A. Finally, as shown for other diseases (53–55), it is possible that copy number variation could affect the relationship between FCGR3A polymorphism and CD risk.

MATERIALS AND METHODS

Human serum samples. Serum samples ($n = 164$) were obtained from MACS, an ongoing prospective study of the natural and treated histories of HIV infection in homosexual and bisexual men living in Baltimore,

Chicago, Pittsburgh, and Los Angeles (29). A total of 6,972 men were enrolled into the MACS between 1984 and 2003. MACS protocols are approved by institutional review boards at each recruitment site, and all participants provided written informed consent. In MACS, CD is diagnosed by positive serum cryptococcal antigen and/or culture.

For the current investigation, 55 HIV-infected men with incident CD (HIV$^+$ CD$^+$) were each matched to two controls: (i) an HIV-infected man without CD (HIV$^+$ CD$^-$) and (ii) an HIV-uninfected man without CD (HIV$^-$ CD$^-$). HIV$^+$ CD$^+$ and HIV$^+$ CD$^-$ men were matched on the following characteristics: (i) age, (ii) recruitment site, (iii) recruitment wave, (iv) CD4$^+$ T cell level ($\pm$50 at the first HIV-positive study visit) (i.e., the enrollment visit for men with prevalent HIV at enrollment and the seroconversion visit for men who contracted HIV during follow-up), (v) the availability of serum samples $\pm$2 years apart, and (vi) the duration of CD-free follow-up time. These stringent matching criteria ensured not only that HIV-infected control men were similar to CD cases with regard to immune status and other characteristics at their first HIV-positive study visit but also that they were also CD-free during the time period in which the cases developed disease. HIV-uninfected controls were matched to CD cases by age, recruitment site, enrollment cohort, and the availability of serum samples $\pm$0.5 years apart. Serum samples obtained for this study were collected a minimum of 1 year prior to CD diagnosis and while the men were off antiretroviral therapy. All sera were received frozen and stored at $-80°$ C until use. Samples were tested in a blinded fashion.

Genomic DNA extraction. Human genomic DNA was isolated from frozen serum samples using a QIAamp DNA blood minikit (Qiagen) following the manufacturer's instructions and per previous reports (56). For some serum samples, DNA was extracted manually using proteinase K and a phenol-chloroform precipitation method (57).

FCGR2A genotyping. The FCGR2A genotypes were determined by PCR and sequencing. FCGR2A displays a G-to-A point mutation in the region specifying its ligand-binding domain, causing an arginine (R; CGT)-to-histidine (H; CAT) amino acid substitution at position 131. The single nucleotide polymorphism (SNP) 131 H/R (rs1801274) was amplified using gene-specific sense 5′-ACCCTTGGAATCTATCCTTAC and antisense 5′-TGAGAAAGCCCATAGCAGCA primers in a 50-μl reaction mixture with 20 ng of genomic DNA, 12.5 pmol each primer, 200 μmol each deoxynucleoside triphosphate (dNTP), and 2.5 U Taq polymerase. PCR conditions were as follows: 10 min of initial denaturation at 95° C followed by 35 cycles at 95° C for 1 min, 62° C for 1 min, and 72° C for 1 min. PCR purification was done using spin columns (Qiagen), and sequencing was performed for both strands (Genewiz). Trace chromatogram files obtained were analyzed using MacVector (with Assembler) software (version 12.0.2; MacVector Inc.).

FCGR3A genotyping. The FCGR3A gene displays a T-to-G substitution at nucleotide 559, resulting in a phenylalanine (F)-to-valine (V) substitution at amino acid position 158. The FCGR3A 158 F/V SNP (rs396991) was determined by nested PCR amplification following a previously published protocol (25). The primers used are listed in Table S1 in the supplemental material. Briefly, gene-specific forward primer 5′ ATA TTTACAGAATGGCACAGG 3′ and reverse primer 5′ GACTTGGTACC CAGGTTGAA 3′ were used in a 25-μl reaction mixture with 20 ng of genomic DNA, 75 ng of each primer, 200 μmol each dNTP, and 0.25 U of Taq polymerase. PCR conditions were as follows: 10 min of initial denaturation at 95° C followed by 35 cycles at 95° C for 1 min, 56° C for 1 min, and 72° C for 1 min. The template of the first round was used in the nested PCR reaction. For the second round, sense 5′ TCATCATAATTCTGACT TCT 3′ and antisense 5′ CTTGAGTGATGGTGATGTTC 3′ primers were used. The PCR components and conditions were as previously described (25). PCR products were purified using spin columns (Qiagen) followed by sequencing in both directions (Genewiz). Trace chromatogram files obtained were analyzed using MacVector (with Assembler) software (version 12.0.2; MacVector Inc.).

Sequenom analysis. A Sequenom MassARRAY iPLEX platform (Genomics Core, Albert Einstein College of Medicine) was used to validate

genotyping results of FCGR2A PCR sequencing. The primers designed (see Table S1 in the supplemental material) annealed at a position directly adjacent to the SNP of interest, and allele-specific extension products were analyzed using mass spectrometry as described in earlier reports (58). Genotypes were assigned using Sequenom software (Sequenom Inc., San Diego, CA).

Statistical analysis of FCGR2A and FCGR3A genotypes. We calculated descriptive statistics for the three study groups and compared the distributions of demographic and clinical characteristics using t tests and Fisher's exact tests. Conditional multivariable logistic regression models were then used to study associations of FCGR polymorphisms with HIV-associated CD. Specifically, we compared HIV^+ CD^+ men to both the combined group of HIV^+ CD^- and HIV^- CD^- men and to HIV^+ CD^- men alone. These regression analyses included adjustment for race/ethnicity (non-Hispanic white versus other) and were conducted under different models of genetic inheritance, including additive, dominant, recessive, and allelic (i.e., effect per additional allele) because assumptions of a specific genetic model of inheritance are rarely justified in association studies (59). Further sensitivity analyses were also conducted by restricting the analyses to non-Hispanic white men and to HIV-positive men alone. Allele frequencies of the studied FCGR genes in the control groups ($n = 109$; HIV^+ CD^- and HIV^- CD^-) were in Hardy-Weinberg equilibrium.

To control for the rate of HIV disease progression in the CD cases and the HIV-infected control men following their matching visits, we used previously published methods to classify men as having a rapid and persistent $CD4^+$ T cell decline after the first HIV-seropositive study visit, having maintained their $CD4^+$ T cell counts, or having a heterogeneous $CD4^+$ T cell decline (60). We then updated our conditional logistic regression analyses to adjust for the rate of $CD4^+$ T cell decline and nadir $CD4^+$ T cell count (<50, 50 to 100, 100 to 200, or >200 cells/μl) in addition to race/ethnicity.

Measurement of total serum IgG1, IgG2, and IgG3 concentrations. Total serum IgG1, IgG2, and IgG3 concentrations were determined using human isotype-specific radial immunodiffusion (RID) kits (The Binding Site, United Kingdom) following the manufacturer's instructions and as described elsewhere (7, 12).

Assessment of serum GXM-IgG levels. GXM-IgG levels were determined as described previously (4–6,17). Briefly, 96-well enzyme-linked immunosorbent assay (ELISA) plates were coated with 10 μg/ml of GXM isolated from *C. neoformans* strain 24067 at 25°C for 3 h and blocked overnight with 1% bovine serum albumin (BSA)–PBS. Heat-inactivated sera were added to the plates at a dilution of 1:30 in 1% BSA–PBS. Bound GXM-IgG was detected using alkaline phosphatase-labeled goat anti-human IgG (Southern Biotechnology) and *p*-nitrophenyl phosphate (Sigma). Absorbance was measured at 405 nm using an ELISA reader (Tecan). A pilot study was done to determine the serum dilution at which to perform the GXM titer measurements. Sera from each group were serially diluted 1:3 in 1% BSA–PBS to find a dilution that fell on the slope of the titration curve. In each group, the dilution of 1:30 fell on the slope of the curve.

***C. neoformans* strains.** Serotype D *C. neoformans*, strain 24067 (American Type Culture Collection), extensively used in mice to study immunity to *C. neoformans* (13, 61), was used. A GFP-labeled 24067 strain was kindly provided by A. Alspaugh (Duke University). Acapsular *C. neoformans* strain CAP67 was used where indicated. For some experiments, *C. neoformans* was heat killed at 65°C for 30 min. *C. neoformans* cultures were grown in Sabouraud's dextrose broth (Becton Dickinson) for 52 to 56 h at 37°C with agitation.

CHO-K1 cell lines. Stably transfected CHO-K1 cell lines expressing either low-affinity 158F or a higher-affinity 158V allotype of FCGR3A were used (31). Cell lines were maintained in minimal essential medium (MEM) (Invitrogen) supplemented with 5% fetal bovine serum (FBS) and 600 μg/ml of hygromycin-B (Invitrogen). To confirm the receptors expressed by the CHO cell lines, murine CD16 monoclonal antibody MEM-154 (ab28091; Abcam) was used (1 μg/ml) followed by the use of 10 μg/ml of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma) as previously described (31). Surface label-

ing of CHO cell lines for binding assays was performed by incubating 10^6 CHO cells (expressing 158F or 158V receptors) with 1 μg/ml of anti-integrin beta 1 antibody (ab115146; Abcam) on ice for 30 min (62, 63). After washing, cells were incubated with 0.5 μg of APC-labeled goat anti-rabbit IgG antibody (sc-3846; Santa Cruz) on ice for 30 min. Cells were washed and proceeded for binding assays.

Assessment of binding of CHO-K1 cell lines to hIgG-*C. neoformans* immune complexes. Serial dilutions of normal human serum (Millipore) (65 mg/ml) as well as purified individual human immunoglobulin isotypes (IgG1, IgG2, IgG3, and IgG4) (The Binding Site) (1 mg/ml) ranging from 1 mg/ml to 0.01 μg/ml were incubated with 10^5 CFU of GFP-expressing *C. neoformans* strain 24067 for 2 h at 37°C to generate immune complexes. Human serum (Millipore) (pooled from multiple donors) was heat inactivated at 56°C for 30 min to eliminate complement activity. The immune complexes were then incubated with APC-labeled CHO-K1 cell lines (expressing 158F or 158V) (10^5 cells/well) in a 96-well plate on ice for 30 min, adapting a previously published protocol (31). No-antibody and no-*C. neoformans* controls were included. Binding was analyzed by scoring GFP^+ APC^+ cells by flow cytometry. Single-color controls were included for proper gating. Data were collected on a BD LSR II flow cytometer (Becton Dickinson, Sunnyvale, CA). A total of 50,000 events per sample were analyzed using FlowJo (Treestar, Ashland, OR).

NK cell lines. Human natural killer (NK) cell lines transduced with either higher-affinity (V) or lower-affinity (f) variants of FCGR3A were obtained from Kerry Campbell at Fox Chase Cancer Center (Philadelphia, PA) (64). The cell lines were maintained in NK-92 culture medium as previously described (65). The parental NK-92 cell line (ATCC CRL-2407) lacking endogenous expression of FCGR3A was also included for measuring the anticryptococcal activity.

Measurement of anticryptococcal activity of NK cell lines. The anticryptococcal activity was assessed by CFU counting as previously described (34). In brief, live *C. neoformans* cells (5×10^3 cells/well) were incubated with FCGR3A polymorphic NK cell lines (effector) at effector/target cell ratios of 20:1, 100:1, and 500:1 at the start of the assay in a total volume of 1 ml in a 48-well plate. Plates were kept at 37°C using 5% CO_2. The number of CFU of *C. neoformans* per well was determined at 0, 24, and 48 h by lysing the effector cells in water followed by dilution and spotting onto Sabouraud's dextrose agar plates in duplicate experiments.

Measurement of NK cell-mediated ADCC. Antibody-dependent cell cytotoxicity was assessed using FCGR3A 158 F/V polymorphic NK cell lines as effector cells and *C. neoformans*-infected human $CD14^+$ $CD16^+$ monocytes as target cells. $CD14^+$ $CD16^+$ monocytes were isolated and purified from human PBMCs as previously described (48). Subsequently, monocytes were infected with *C. neoformans* strain 24067 at a ratio of 1:10 in the presence of 20% heat-inactivated human serum (Millipore) at 37°C and 5% CO_2 for 2 h (66). Extracellular cryptococcus was removed by extensive washing. Infected monocytes (target; 1×10^4) were then incubated with NK cell lines (effector; 1×10^5) at an effector-to-target ratio of 10:1 for 4 h at 37°C and 5% CO_2 in the presence of (i) 10 μg/ml of normal human serum (Millipore) (heat inactivated) or (ii) 10 μg/ml of total IgG (IgG1 plus IgG2 plus IgG3 plus IgG4; The Binding Site, United Kingdom). A no-antibody control was also included. After 4 h, the supernatant were collected and assayed for lactate dehydrogenase (LDH) production, as previously described (67), using a Cytotox 96 nonradioactive cytotoxicity assay (Promega). Percent cytotoxicity was calculated per the manufacturer's instructions. After analyzing the supernatants, the remaining cells were lysed in water, followed by dilution and spotting onto Sabouraud's dextrose agar plates, in order to assess fungal burden.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00573-13/-/DCSupplemental.

Figure S1, TIFF file, 1.3 MB.
Table S1, DOC file, 0.1 MB.

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REFERENCES

1. **Datta K, Pirofski LA.** 2006. Towards a vaccine for Cryptococcus neoformans: principles and caveats. FEMS Yeast Res. **6**:525–536.
2. **Jarvis JN, Lawn SD, Vogt M, Bangani N, Wood R, Harrison TS.** 2009. Screening for cryptococcal antigenemia in patients accessing an antiretroviral treatment program in South Africa. Clin. Infect. Dis. **48**:856–862.
3. **Chayakulkeeree M, Perfect JR.** 2006. Cryptococcosis. Infect. Dis. Clin. North Am. **20**:507–544, v–vi.
4. **Fleuridor R, Lyles RH, Pirofski L.** 1999. Quantitative and qualitative differences in the serum antibody profiles of human immunodeficiency virus-infected persons with and without Cryptococcus neoformans meningitis. J. Infect. Dis. **180**:1526–1535.
5. **Deshaw M, Pirofski LA.** 1995. Antibodies to the Cryptococcus neoformans capsular glucuronoxylomannan are ubiquitous in serum from HIV+ and HIV− individuals. Clin. Exp. Immunol. **99**:425–432.
6. **Abadi J, Pirofski LA.** 1999. Antibodies reactive with the cryptococcal capsular polysaccharide glucuronoxylomannan are present in sera from children with and without human immunodeficiency virus infection. J. Infect. Dis. **180**:915–919.
7. **Subramaniam K, French N, Pirofski LA.** 2005. Cryptococcus neoformans-reactive and total immunoglobulin profiles of human immunodeficiency virus-infected and uninfected Ugandans. Clin. Diagn. Lab. Immunol. **12**:1168–1176.
8. **Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM.** 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23**:525–530.
9. **Pappas PG.** 2013. Cryptococcal infections in non-HIV-infected patients. Trans. Am. Clin. Climatol. Assoc. **124**:61–79.
10. **Pyrgos V, Seitz AE, Steiner CA, Prevots DR, Williamson PR.** 2013. Epidemiology of cryptococcal meningitis in the US: 1997–2009. PLoS One **8**:e56269. doi: 10.1371/journal.pone.0056269.
11. **Singh N, Alexander BD, Lortholary O, Dromer F, Gupta KL, John GT, del Busto R, Klintmalm GB, Somani J, Lyon GM, Pursell K, Stosor V, Muñoz P, Limaye AP, Kalil AC, Pruett TL, Garcia-Diaz J, Humar A, Houston S, House AA, Wray D, Orloff S, Dowdy LA, Fisher RA, Heitman J, Wagener MM, Husain S.** 2008. Pulmonary cryptococcosis in solid organ transplant recipients: clinical relevance of serum cryptococcal antigen. Clin. Infect. Dis. **46**:e12–e18.
12. **Subramaniam K, Metzger B, Hanau LH, Guh A, Rucker L, Badri S, Pirofski LA.** 2009. IgM(+) memory B cell expression predicts HIV-associated cryptococcosis status. J. Infect. Dis. **200**:244–251.
13. **Rohatgi S, Pirofski LA.** 2012. Molecular characterization of the early B cell response to pulmonary Cryptococcus neoformans infection. J. Immunol. **189**:5820–5830.
14. **Szymczak W, Davis M, Lundy S, Dufaud C, Olszewski M, Pirofski L.** 2013. X-linked immunodeficient mice exhibit enhanced susceptibility to Cryptococcus neoformans infection. mBio **4**:e00265-13. doi: 10.1128/mBio.00265-13.
15. **Chen LC, Goldman DL, Doering TL, Pirofski L, Casadevall A.** 1999. Antibody response to Cryptococcus neoformans proteins in rodents and humans. Infect. Immun. **67**:2218–2224.
16. **Goldman DL, Khine H, Abadi J, Lindenberg DJ, Pirofski L, Niang R, Casadevall A.** 2001. Serologic evidence for Cryptococcus neoformans infection in early childhood. Pediatrics **107**:E66. doi: 10.1542/peds.107.5.e66.
17. **Mukherjee S, Lee SC, Casadevall A.** 1995. Antibodies to Cryptococcus neoformans glucuronoxylomannan enhance antifungal activity of murine macrophages. Infect. Immun. **63**:573–579.
18. **Zhong Z, Pirofski LA.** 1996. Opsonization of Cryptococcus neoformans by human anticryptococcal glucuronoxylomannan antibodies. Infect. Immun. **64**:3446–3450.
19. **Casadevall A, Pirofski L.** 2005. Insights into mechanisms of antibody-mediated immunity from studies with Cryptococcus neoformans. Curr. Mol. Med. **5**:421–433.
20. **Casadevall A, Cleare W, Feldmesser M, Glatman-Freedman A, Goldman DL, Kozel TR, Lendvai N, Mukherjee J, Pirofski LA, Rivera J, Rosas AL, Scharff MD, Valadon P, Westin K, Zhong Z.** 1998. Characterization of a murine monoclonal antibody to Cryptococcus neoformans polysaccharide that is a candidate for human therapeutic studies. Antimicrob. Agents Chemother. **42**:1437–1446.
21. **Nimmerjahn F, Ravetch JV.** 2006. Fcgamma receptors: old friends and new family members. Immunity **24**:19–28.
22. **Yuan R, Clynes R, Oh J, Ravetch JV, Scharff MD.** 1998. Antibody-mediated modulation of Cryptococcus neoformans infection is dependent on distinct Fc receptor functions and IgG subclasses. J. Exp. Med. **187**:641–648.
23. **Beenhouwer DO, Yoo EM, Lai CW, Rocha MA, Morrison SL.** 2007. Human immunoglobulin G2 (IgG2) and IgG4, but not IgG1 or IgG3, protect mice against Cryptococcus neoformans infection. Infect. Immun. **75**:1424–1435.
24. **van Sorge NM, van der Pol WL, van de Winkel JG.** 2003. FcgammaR polymorphisms: implications for function, disease susceptibility and immunotherapy. Tissue Antigens **61**:189–202.
25. **Meletiadis J, Walsh TJ, Choi EH, Pappas PG, Ennis D, Douglas J, Pankey GA, Larsen RA, Hamill RJ, Chanock S.** 2007. Study of common functional genetic polymorphisms of FCGR2A, 3A and 3B genes and the risk for cryptococcosis in HIV-uninfected patients. Med. Mycol. **45**:513–518.
26. **Hu XP, Wu JQ, Zhu LP, Wang X, Xu B, Wang RY, Ou XT, Weng XH.** 2012. Association of Fcγ receptor IIB polymorphism with cryptococcal meningitis in HIV-uninfected Chinese patients. PLoS One **7**:e42439. doi: 10.1371/journal.pone.0042439.
27. **Lopez-Escamez JA, Saenz-Lopez P, Gazquez I, Moreno A, Gonzalez-Oller C, Soto-Varela A, Santos S, Aran I, Perez-Garrigues H, Ibanez A, Lopez-Nevot MA.** 2011. Polymorphisms of CD16A and CD32 Fcgamma receptors and circulating immune complexes in Meniere's disease: a case-control study. BMC Med. Genet. **12**:2. doi: 10.1186/1471-2350-12-2.
28. **Wu J, Edberg JC, Redecha PB, Bansal V, Guyre PM, Coleman K, Salmon JE, Kimberly RP.** 1997. A novel polymorphism of FcgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. J. Clin. Invest. **100**:1059–1070.
29. **Kaslow RA, Ostrow DG, Detels R, Phair JP, Polk BF, Rinaldo CR, Jr.** 1987. The multicenter AIDS cohort study: rationale, organization, and selected characteristics of the participants. Am. J. Epidemiol. **126**:310–318.
30. **Poonia B, Kijak GH, Pauza CD.** 2010. High affinity allele for the gene of FCGR3A is risk factor for HIV infection and progression. PLoS One **5**:e15562. doi: 10.1371/journal.pone.0015562.
31. **Armour KL, Smith CS, Clark MR.** 2010. Expression of human FcgammaRIIIa as a GPI-linked molecule on CHO cells to enable measurement of human IgG binding. J. Immunol. Methods **354**:20–33.
32. **Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M.** 1997. Fc gammaRIIIa-158V/F polymorphism influences the binding of

IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood **90:**1109–1114.

33. **Lipscomb MF, Alvarellos T, Toews GB, Tompkins R, Evans Z, Koo G, Kumar V.** 1987. Role of natural killer cells in resistance to Cryptococcus neoformans infections in mice. Am. J. Pathol. **128:**354–361.

34. **Marr KJ, Jones GJ, Zheng C, Huston SM, Timm-McCann M, Islam A, Berenger BM, Ma LL, Wiseman JC, Mody CH.** 2009. Cryptococcus neoformans directly stimulates perforin production and rearms NK cells for enhanced anticryptococcal microbicidal activity. Infect. Immun. **77:** 2436–2446.

35. **Houpt DC, Pfrommer GS, Young BJ, Larson TA, Kozel TR.** 1994. Occurrences, immunoglobulin classes, and biological activities of antibodies in normal human serum that are reactive with Cryptococcus neoformans glucuronoxylomannan. Infect. Immun. **62:**2857–2864.

36. **Alvarez M, Casadevall A.** 2006. Phagosome extrusion and host-cell survival after Cryptococcus neoformans phagocytosis by macrophages. Curr. Biol. **16:**2161–2165.

37. **García-Rodas R, Zaragoza O.** 2012. Catch me if you can: phaocytosis and killing avoidance by Cryptococcus neoformans. FEMS Immunol. Med. Microbiol. **64:**147–161.

38. **Casadevall A.** 2012. Amoeba provide insight into the origin of virulence in pathogenic fungi. Adv. Exp. Med. Biol. **710:**1–10.

39. **Ancuta P, Wang J, Gabuzda D.** 2006. CD16+ monocytes produce IL-6, CCL2, and matrix metalloproteinase-9 upon interaction with CX3CL1-expressing endothelial cells. J. Leukoc. Biol. **80:**1156–1164.

40. **Eugenin EA, Osiecki K, Lopez L, Goldstein H, Calderon TM, Berman JW.** 2006. CCL2/monocyte chemoattractant protein-1 mediates enhanced transmigration of human immunodeficiency virus (HIV)-infected leukocytes across the blood-brain barrier: a potential mechanism of HIV-CNS invasion and NeuroAIDS. J. Neurosci. **26:**1098–1106.

41. **Ziegler-Heitbrock L.** 2007. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J. Leukoc. Biol. **81:**584–592.

42. **Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F.** 2009. Evidence of a role for monocytes in dissemination and brain invasion by Cryptococcus neoformans. Infect. Immun. **77:**120–127.

43. **Pinheiro da Silva F, Aloulou M, Skurnik D, Benhamou M, Andremont A, Velasco IT, Chiamolera M, Verbeek JS, Launay P, Monteiro RC.** 2007. CD16 promotes Escherichia coli sepsis through an FcR gamma inhibitory pathway that prevents phagocytosis and facilitates inflammation. Nat. Med. **13:**1368–1374.

44. **Lehrnbecher T, Foster CB, Zhu S, Leitman SF, Goldin LR, Huppi K, Chanock SJ.** 1999. Variant genotypes of the low-affinity Fcgamma receptors in two control populations and a review of low-affinity Fcgamma receptor polymorphisms in control and disease populations. Blood **94:** 4220–4232.

45. **Brown EE, Fallin MD, Goedert JJ, Chen R, Whitby D, Foser CB, Lauria C, Alberg AJ, Messina A, Montella M, Rezza G, Vitale F, Chanock SJ, Kaposi Sarcoma Genetics Working Group.** 2005. A common genetic variant in FCGR3A-V158F and risk of Kaposi sarcoma herpesvirus infection and classic Kaposi sarcoma. Cancer Epidemiol. Biomarkers Prev. **14:** 633–637.

46. **Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H.** 2002. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood **99:**754–758.

47. **Mailliard RB, Son YI, Redlinger R, Coates PT, Giermasz A, Morel PA, Storkus WJ, Kalinski P.** 2003. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. J. Immunol. **171:**2366–2373.

48. **Buckner CM, Calderon TM, Willams DW, Belbin TJ, Berman JW.** 2011. Characterization of monocyte maturation/differentiation that facilitates their transmigration across the blood-brain barrier and infection by HIV: implications for NeuroAIDS. Cell. Immunol. **267:**109–123.

49. **Forthal DN, Gabriel EE, Wang A, Landucci G, Phan TB.** 2012. Association of Fcγ receptor IIIa genotype with the rate of HIV infection after gp120 vaccination. Blood **120:**2836–2842.

50. **Hajjeh RA, Conn LA, Stephens DS, Baughman W, Hamill R, Graviss E, Pappas PG, Thomas C, Reingold A, Rothrock G, Hutwagner LC, Schuchat A, Brandt ME, Pinner RW.** 1999. Cryptococcosis: population-based multistate active surveillance and risk factors in human immunodeficiency virus-infected persons. Cryptococcal Active Surveillance Group. J. Infect. Dis. **179:**449–454.

51. **Visnegarwala F, Graviss EA, Lacke CE, Dural AT, Johnson PC, Atmar RL, Hamill RJ.** 1998. Acute respiratory failure associated with cryptococcosis in patients with AIDS: analysis of predictive factors. Clin. Infect. Dis. **27:**1231–1237.

52. **Monari C, Kozel TR, Paganelli F, Pericolini E, Perito S, Bistoni F, Casadevall A, Vecchiarelli A.** 2006. Microbial immune suppression mediated by direct engagement of inhibitory Fc receptor. J. Immunol. **177:** 6842–6851.

53. **Niederer HA, Willcocks LC, Rayner TF, Yang W, Lau YL, Williams TN, Scott JA, Urban BC, Peshu N, Dunstan SJ, Hien TT, Phu NH, Padyukov L, Gunnarsson I, Svenungsson E, Savage CO, Watts RA, Lyons PA, Clayton DG, Smith KG.** 2010. Copy number, linkage disequilibrium and disease association in the FCGR locus. Hum. Mol. Genet. **19:**3282–3294.

54. **Aitman TJ, Dong R, Vyse TJ, Norsworthy PJ, Johnson MD, Smith J, Mangion J, Roberton-Lowe C, Marshall AJ, Petretto E, Hodges MD, Bhangal G, Patel SG, Sheehan-Rooney K, Duda M, Cook PR, Evans DJ, Domin J, Flint J, Boyle JJ, Pusey CD, Cook HT.** 2006. Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans. Nature **439:**851–855.

55. **Fanciulli M, Norsworthy PJ, Petretto E, Dong R, Harper L, Kamesh L, Heward JM, Gough SC, de Smith A, Blakemore AI, Froguel P, Owen CJ, Pearce SH, Teixeira L, Guillevin L, Graham DS, Pusey CD, Cook HT, Vyse TJ, Aitman TJ.** 2007. FCGR3B copy number variation is associated with susceptibility to systemic, but not organ-specific, autoimmunity. Nat. Genet. **39:**721–723.

56. **Bucasas KL, Pandya GA, Pradhan S, Fleischmann RD, Peterson SN, Belmont JW.** 2009. Assessing the utility of whole-genome amplified serum DNA for array-based high throughput genotyping. BMC Genet. **10:** 85. doi: 10.1186/1471-2156-10-85.

57. **Maaroufi Y, Ahariz N, Husson M, Crokaert F.** 2004. Comparison of different methods of isolation of DNA of commonly encountered Candida species and its quantitation by using a real-time PCR-based assay. J. Clin. Microbiol. **42:**3159–3163.

58. **Joshi D, Harris NB, Waters R, Thacker T, Mathema B, Krieswirth B, Sreevatsan S.** 2012. Single nucleotide polymorphisms in the Mycobacterium bovis genome resolve phylogenetic relationships. J. Clin. Microbiol. **50:**3859–3861.

59. **Salanti G, Southam L, Altshuler D, Ardlie K, Barroso I, Boehnke M, Cornelis MC, Frayling TM, Grallert H, Grarup N, Groop L, Hansen T, Hattersley AT, Hu FB, Hveem K, Illig T, Kuusisto J, Laakso M, Langenberg C, Lyssenko V, McCarthy MI, Morris A, Morris AD, Palmer CN, Payne F, Platou CG, Scott LJ, Voight BF, Wareham NJ, Zeggini E, Ioannidis JP.** 2009. Underlying genetic models of inheritance in established type 2 diabetes associations. Am. J. Epidemiol. **170:**537–545.

60. **Kuniholm MH, Gao X, Xue X, Kovacs A, Anastos K, Marti D, Greenblatt RM, Cohen MH, Minkoff H, Gange SJ, Fazzari M, Young MA, Strickler HD, Carrington M.** 2011. Human leukocyte antigen genotype and risk of HIV disease progression before and after initiation of antiretroviral therapy. J. Virol. **85:**10826–10833.

61. **Subramaniam KS, Datta K, Quintero E, Manix C, Marks MS, Pirofski LA.** 2010. The absence of serum IgM enhances the susceptibility of mice to pulmonary challenge with Cryptococcus neoformans. J. Immunol. **184:** 5755–5767.

62. **Bretscher MS.** 1989. Endocytosis and recycling of the fibronectin receptor in CHO cells. EMBO J. **8:**1341–1348.

63. **Zhang Z, Morla AO, Vuori K, Bauer JS, Juliano RL, Ruoslahti E.** 1993. The alpha v beta 1 integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin. J. Cell Biol. **122:**235–242.

64. **Binyamin L, Alpaugh RK, Hughes TL, Lutz CT, Campbell KS, Weiner LM.** 2008. Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. J. Immunol. **180:**6392–6401.

65. **Gong JH, Maki G, Klingemann HG.** 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia **8:**652–658.

66. **Alvarez M, Burn T, Luo Y, Pirofski LA, Casadevall A.** 2009. The outcome of Cryptococcus neoformans intracellular pathogenesis in human monocytes. BMC Microbiol. **9:**51. doi: 10.1186/1471-2180-9-51.

67. **Chow SK, Smith C, MacCarthy T, Pohl MA, Bergman A, Casadevall A.** 2013. Disease-enhancing antibodies improve the efficacy of bacterial toxin-neutralizing antibodies. Cell Host Microbe **13:**417–428.