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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 158VV 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.
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–IgG 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 (rs969991), 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, recruitment 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 genotype without the 158F allele, an OR of 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
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 = 0.005) but the FCGR3A 158F V 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 1: Characteristics of the study population

<table>
<thead>
<tr>
<th>Study participant characteristic</th>
<th>HIV+ CD+ (n = 55)</th>
<th>HIV+ CD− (n = 54)</th>
<th>HIV− CD− (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (IQR) age</td>
<td>34 (29–37)</td>
<td>34 (30–37)</td>
<td>35 (31–45)</td>
</tr>
<tr>
<td>No. with race/ethnicity:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White non-Hispanic</td>
<td>43</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>Otherb</td>
<td>12</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>No. with yr of first HIV-positive study visit:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1984–1986</td>
<td>45</td>
<td>44</td>
<td>NA</td>
</tr>
<tr>
<td>1987–1990</td>
<td>10</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>No. with CD4+ count at first HIV-positive visit (cells/µl):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;200</td>
<td>1</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>200–500</td>
<td>16</td>
<td>13</td>
<td>NA</td>
</tr>
<tr>
<td>&gt;500</td>
<td>38</td>
<td>39</td>
<td>NA</td>
</tr>
<tr>
<td>Median (IQR) CD4+ nadir (cells/µl)c</td>
<td>42 (16–133)</td>
<td>75 (16–295)</td>
<td>NA</td>
</tr>
<tr>
<td>No. with CD4+ nadir (cells/µl):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>29</td>
<td>22</td>
<td>NA</td>
</tr>
<tr>
<td>50–99</td>
<td>9</td>
<td>6</td>
<td>NA</td>
</tr>
<tr>
<td>100–199</td>
<td>8</td>
<td>9</td>
<td>NA</td>
</tr>
<tr>
<td>200+</td>
<td>9</td>
<td>17</td>
<td>NA</td>
</tr>
<tr>
<td>No. with yr of first HAART:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1993–1996</td>
<td>54</td>
<td>42</td>
<td>NA</td>
</tr>
<tr>
<td>1997–2000</td>
<td>1</td>
<td>11</td>
<td>NA</td>
</tr>
<tr>
<td>2001–2005</td>
<td>0</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>No. with clinical AIDSd</td>
<td>55</td>
<td>32</td>
<td>NA</td>
</tr>
<tr>
<td>Median (IQR) no. of yrs from first HIV-positive visit to clinical AIDS e</td>
<td>5.8 (3.8–7.4)</td>
<td>8.3 (6.5–9.5)</td>
<td>NA</td>
</tr>
</tbody>
</table>

### Table 2: Fc gamma receptor genotype frequencies in the study population

<table>
<thead>
<tr>
<th>Study population</th>
<th>No. (and/or %) of participants with each genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FCGR3A 158 F/V</td>
</tr>
<tr>
<td>All participants (n = 164)</td>
<td>FF</td>
</tr>
<tr>
<td></td>
<td>FV</td>
</tr>
<tr>
<td></td>
<td>VV</td>
</tr>
<tr>
<td></td>
<td>HR</td>
</tr>
<tr>
<td>Caucasiansa</td>
<td>(50)</td>
</tr>
<tr>
<td>African Americansb</td>
<td>(42)</td>
</tr>
</tbody>
</table>

### Notes

a. Genotype frequencies in normal healthy U.S. Caucasians (44).
was also no significant difference in total serum IgG1, IgG2, IgG3, or GXM-IgG levels between the HIV/H11001_CD/H11001 and HIV/H11001_CD/H11002 groups (Fig. 1). However, each HIV-infected (HIV/H11001_CD/H11001 and HIV/H11001_CD/H11002) group had more serum IgG1 and IgG3 and less IgG2 than the HIV-uninfected (HIV/H11002_CD/H11002) 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>
<thead>
<tr>
<th>Parameter</th>
<th>No. of men</th>
<th>Value(s) for indicated inheritance model</th>
<th>Additive</th>
<th>Dominant</th>
<th>Recessive</th>
<th>Allelic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 109)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>FCGR3A 158 F/V genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF</td>
<td>37</td>
<td>3.3 (1.4–7.9)</td>
<td>0.007</td>
<td>2.9 (1.0–8.4)</td>
<td>0.06</td>
<td>17.6 (2.1–148.2)</td>
</tr>
<tr>
<td>FV</td>
<td>57</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.7 (0.5–5.5)</td>
</tr>
<tr>
<td>VV</td>
<td>15</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>20.9 (2.5–177.3)</td>
</tr>
<tr>
<td>CD4+ status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable CD4+ count</td>
<td>34</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Heterogeneous CD4+ decline</td>
<td>62</td>
<td>1.4 (0.4–4.9)</td>
<td>0.56</td>
<td>1.8 (0.6–5.7)</td>
<td>0.31</td>
<td>1.4 (0.4–5.2)</td>
</tr>
<tr>
<td>Rapid CD4+ decline</td>
<td>13</td>
<td>31.6 (2.3–438.2)</td>
<td>0.01</td>
<td>24.3 (2.3–263.6)</td>
<td>0.008</td>
<td>48.6 (2.9–803.7)</td>
</tr>
<tr>
<td>CD4+ nadir</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;200</td>
<td>26</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>100–200</td>
<td>17</td>
<td>1.3 (0.2–8.3)</td>
<td>0.78</td>
<td>1.1 (0.2–6.4)</td>
<td>0.89</td>
<td>0.9 (0.1–6.7)</td>
</tr>
<tr>
<td>50–100</td>
<td>15</td>
<td>5.3 (0.8–34.1)</td>
<td>0.08</td>
<td>5.0 (0.8–30.1)</td>
<td>0.08</td>
<td>4.0 (0.7–23.4)</td>
</tr>
<tr>
<td>&lt;50</td>
<td>51</td>
<td>6.1 (1.0–35.0)</td>
<td>0.04</td>
<td>4.2 (0.9–19.7)</td>
<td>0.07</td>
<td>5.8 (0.9–37.9)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White non-Hispanic</td>
<td>91</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Other race/ethnicity</td>
<td>18</td>
<td>9.0 (1.2–65.5)</td>
<td>0.03</td>
<td>6.2 (1.0–37.7)</td>
<td>0.05</td>
<td>15.6 (1.8–133.3)</td>
</tr>
</tbody>
</table>

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.
FCGR3A Polymorphism in Cryptococcosis

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+ monocyes (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 cell 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.
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
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 the monocyte/macrophage C. neoformans hIgG complexes bound to CD14+ monocytes 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 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 158F/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 caveat 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 Kaposis’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 dissemination to the brain. In support of 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 158F/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 caveat 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).

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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 158V 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 158V 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 GMX binds this receptor and induces an inhibitory signal to macrophages (52), it is possible that differing affinities of GMX-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 (≥ 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 ± 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 ± 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′-ACCCCTGGGAATCTATCCTTAC and antisense 5′-TGAGAAAGCCCATACGAGCA 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 V/F 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 TTACAGAATGGAACAGG 3’ and reverse primer 5’ GACTTGGTACC CAGGGTGA 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, 62°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’ TCTACATAATTCTGACT TCT 3’ and antisense 5’ CTTGAGATGTGAGTTCTC 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 CD4+ T cell decline. Specifically, we compared HIV+ 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 radiolabelled immunosorbent assay (ELISA) plates 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 with 1% bovine serum albumin (BSA)–PBS. Heat-inactivated sera were also conducted by restricting the analyses to non-Hispanic white men and to HIV-positive men alone. Allele frequencies of the studied FCGR types (IgG1, IgG2, IgG3, and IgG4) (The Binding Site) (1 mg/ml) ranging (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 106 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 36°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) (106 cells/well) in a 96-well plate on ice for 30 min, after 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 LSRII 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 (I) 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 anticytotoxic activity.

Measurement of anticytotoxic activity of NK cell lines. The anticytotoxic activity was assessed by CFU counting as previously described (34). In brief, live C. neoformans cells (5 × 105 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% CO2. The number of CFU of C. neoformans per well was determined at 0, 24, and 48 h by lysing the effector cells in water following 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% CO2 for 2 h (66). Extracellular cryptococcus was removed by extensive washing. Infected monocytes (target; 1 × 105) were then incubated with NK cell lines (effector; 1 × 104) at an effector-to-target ratio of 10:1 for 4 h at 37°C and 5% CO2 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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Elaboration of Pathology through income that was derived from commercial exploitation of patented antibodies.

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