Mucin 1 (MUC1) is a membrane-bound glycoprotein expressed at low levels by healthy tissues but overexpressed and aberrantly glycosylated in the majority of adenocarcinomas. Among 75 distinct tumor-associated antigens, MUC1 has been ranked second as a priority vaccine target by the NCI Translational Research Working Group. For designing an effective MUC1-based active (vaccine) or passive (antibody) immunotherapy, and for a proper evaluation of the therapeutic efficacy of these interventions in clinical trials, further insights into the host factors that contribute to MUC1-targeting immune responses are required. Naturally occurring anti-MUC1 antibodies are associated with improved prognosis in patients affected by several adenocarcinomas. Antibody-dependent cell-mediated cytotoxicity (ADCC) is one of the major mechanisms underlying the clinical efficacy of anticancer monoclonal antibodies (mAbs), such as the mucin 1 (MUC1)-targeting molecule HuHMFG1. IgG antibodies trigger ADCC upon interaction with Fcγ receptors (FcγRs) expressed on the surface of immune effector cells. Polymorphisms affecting FcγRs are known to influence the magnitude of ADCC, but the impact of natural genetic variations in the Fc-coding sequence, γ marker (GM) allotypes, has not been adequately investigated. Using an ADCC inhibition assay, we demonstrate that IgG1 antibodies of the 3+, 1−, 2− GM allotype block almost all valine-containing FcγRIIIa receptors expressed by natural killer (NK) cells, inhibiting by 93% their ability to mediate HuHMFG1-dependent ADCC against DU145 prostate cancer cells. Of note, the ADCC-inhibitory effect of the same IgG1 molecules was significantly reduced when NK cells expressed phenylalanine-containing FcγRIIIa (93% vs. 50%; P = 0.0000005). These and other findings presented here have important therapeutic implications for the use of anti-MUC1 mAbs in patients with prostate cancer and other MUC1-overexpressing adenocarcinomas.
human prostate cancer DU145 cells. In this context, we used 3 allotypically different IgG1 molecules to inhibit the ADCC response of NK cells against DU145 cells elicited by a humanized MUC1-targeting IgG1 mAb, HuHMFG1. In such an ADCC inhibition assay, anti-MUC1 HuHMFG1 antibodies bound to target DU145 cells compete with allotypically disparate IgG1 molecules for binding to activating FcγRIIIA receptors expressed by NK cells.

Serum samples from healthy blood donors were allotyped for all four known IgG1 allotypes—GM 1/a, 2/x, 3/f, and 17/z—by standard hemagglutination-inhibition assays. Allotypes 3 and 17 affect the Fd region of γ1 chains, whereas 1 and 2 affect their Fc fragment. Total IgGs from the pooled sera of 10 subjects expressing the GM allotype 3+,1-,2-, 10 individuals expressing the GM allotype 17+,1+,2-, and 10 people expressing the GM allotype 17+,1+,2- were concentrated by ammonium sulfate fractionation. IgG1 proteins were then isolated by subclass-specific affinity chromatography. FCGR3A genotyping was performed by real-time PCR (RT-PCR), using a pre-designed TaqMan® genotyping assay from Applied Biosystems Inc. NK cells were isolated from peripheral blood mononuclear cells (PBMCs) by affinity depletion of non-NK cells, using a kit from Miltenyi Biotec, according to the manufacturer’s protocol. ADCC assays were performed by a technique modified from Macdonald et al., using the Cytotox-96 kit from Promega Corporation, which quantify lactate dehydrogenase (LDH) activity. The spontaneous release of LDH from target cells incubated with NK cells—possibly due to killer-cell immunoglobulin-like receptor (KIR)-dependent cytotoxicity—was used as blank (negative control). Relative ADCC inhibition was calculated according to the formula: ADCC inhibition (%) = 100 × (Control LDH activity – Test LDH activity) / (Control LDH activity); where Test consists of DU145 target cells incubated with aggregated IgG1 of defined GM allotype, HuHMFG1 antibodies, and NK cells, while (positive) Control consists of DU145 cells incubated with HuHMFG1 antibodies and NK cells only. Results are expressed as means and standard deviations of 7 experimental replications. Employing the Excel (Microsoft) statistical package, one-way ANOVA was used to compare the percentage of ADCC inhibition associated with specific GM-FcγRIIIA combinations. All tests were two-tailed, and the threshold for statistical significance was set to \( P < 0.05 \).

As shown in Table 1, the inhibitory effect of all 3 genetically disparate IgG1 antibodies on HuHMFG1-dependent NK cell-mediated ADCC against DU145 prostate cancer cells was similar when NK cells expressed the phenylalanine (F)-containing variant of FcγRIIIa, whereas highly significant differences were observed in the presence of NK cells expressing valine (V)-containing FcγRIIIa receptors. Thus, at a concentration of 25 µg/mL, IgG1 molecules of the GM 3+,1,2- allotype blocked almost all V-containing FcγRIIIa receptors expressed on the surface of NK cells, resulting in the inhibition of HuHMFG1-dependent ADCC against DU145 prostate cancer cells by 93%. Conversely, the inhibitory effect of the same IgG1 molecules was significantly reduced when NK cells expressed F-containing FcγRIIIa receptors (93% vs. 50%; \( P = 0.0000005 \)). Of note, IgG1 antibodies of the GM 17+,1+,2- allotype similarly discriminated between the 2 FcγRIIIA genotypes (88 vs. 44%; \( P = 0.0002 \)). In contrast, IgG1 molecules of the GM allotype 17+,1+,2- allotype similarly discriminated against the 2 FcγRIIIA genotypes (88 vs. 44%; \( P = 0.0002 \)).

The results presented here show distinct epistatic interactions between the GM allotype of IgG1 molecules and FcγRIIIA variants on the ability of NK cells to mediate ADCC against prostate cancer cells, being performed with important therapeutic implications for the use of anti-MUC1 mAbs in patients with prostate cancer and other MUC1-overexpressing adenocarcinomas. The role of ADCC in the therapeutic efficacy of anticancer mAbs is well recognized, and a great deal of effort is currently being directed at engineering Fc variants with optimized affinity for activating and inhibiting FcγRs. The results described here suggest that, along with these efforts, the role of naturally occurring Fc variants, which have been maintained throughout our evolutionary history, in ADCC and other Fc-mediated immunosurveillance mechanisms should be carefully evaluated.

Of note, the magnitude of another Fc-dependent immunosurveillance mechanism—complement-dependent cytotoxicity (CDC)—may also be influenced by GM allotypes. CDC contributes to the efficacy of some anticancer mAbs, including the anti-CD20 mAb rituximab and the anti-CD52 mAb alemtuzumab. Clq, which triggers the complement cascade, binds slightly better to IgG3 proteins bearing the GM 21 than to those of the alternative GM allotype 5. This suggests that IgG3

### Table 1. Inhibition of HuHMFG1-dependent NK cell-mediated ADCC by different FCGR3A genotypes in the presence of allotypically disparate IgG1 antibodies

| IgG1 allotype | FCGR3A variants | Inhibition of ADCC (%) | \( P \) value |
|--------------|-----------------|------------------------|-------------|
| GM 3+,1,2-   | VV              | 92.87 ± 8.61%          | 0.00000046  |
|              | FF              | 50.41 ± 2.94%          |             |
| GM 17+,1+,2- | VV              | 31.04 ± 20.65%         | 0.15        |
|              | FF              | 48.56 ± 18.24%         |             |
| GM 17+,1+,2- | VV              | 87.70 ± 6.72%          | 0.00018     |
|              | FF              | 43.86 ± 17.32%         |             |

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; FCGR3A, Fc fragment of IgG, low affinity IIIa receptor (CD16a); NK, natural killer.
mAbs bearing the GM 21 in their Fc region are probably more effective in triggering CDC than those of the GM allotype 5.

As mentioned above, MUC1 is a prominent target for cancer immunotherapy. However, the clinical trials involving MUC1-targeting humanized mAbs such as HuHMFG-1 performed to date have failed. New therapeutic strategies are therefore urgently needed. Evaluating the impact of all 18 serologically-determined GM specificities and FCGR2A and FCGR3A alleles in the ability of IgG molecules to trigger ADCC and CDC may provide novel insights toward this endeavor. In addition, the results of such studies may identify the putative mechanism(s) underlying the involvement of these genes in the development of prostate cancer.

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In summary, this is the first report showing the epistatic contributions of GM allotypes and FcγRIIIa variants to the ability of NK cells to mediate ADCC against prostate cancer cells. Our findings must be confirmed by independent investigations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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