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Murine IgG1 and IgG3 Isotype Switch Variants Promote Phagocytosis of Cryptococcus neoformans through Different Receptors

Carolyn A. Saylor,* Ekaterina Dadachova,*† and Arturo Casadevall*‡

Almost 3 decades ago, murine IgG3 was proposed to interact with a different receptor than the other IgG subclasses, but the issue remains unresolved. The question of whether a specific receptor exists for IgG3 is critically important for understanding Ab-mediated immunity against Cryptococcus neoformans, where the different isotypes manifest profound differences in protective efficacy. In this study, we revisited this question by analyzing IgG1- and IgG3-mediated phagocytosis with variable region-identical mAbs using mouse macrophages deficient in various receptors and in conditions of FcγR and complement receptor blockage with specific Abs. IgG3 was an efficient opsonin for C. neoformans in FcγR- and CD18-deficient cells and in the presence of blocking Abs to FcγR and complement receptor. Like IgG1, IgG3-mediated phagocytosis was associated with fungal residence in a mature phagosome that was followed by intracellular replication and exocytosis events. We conclude that a specific receptor for IgG3 exists in mice that is structurally different from the known FcγR.

Phagocytosis is a receptor-mediated event in which the specific recognition of microbes by phagocytic cells, such as macrophages or dendritic cells, leads to microbial internalization and targeting to a phagosomal compartment for degradation and Ag presentation (1–3). During an adaptive immune response, Ab is the primary mediator of this interaction; microbes bound by specific IgG interact with FcγRs on effector cells to promote clearance of infection (4, 5). Characterizing the receptor interactions during Ab-mediated phagocytosis is important for understanding the role of Ab generated during host defense as well as for assessing the mechanism of passive Ab therapy, by which treatment with immune serum or specific mAbs can ameliorate disease (6–8).

In mice, the activating FcγRs are FcγRI, FcγRIII, and FcγRIV, all of which share a common γ-chain containing an intracellular ITAM sequence necessary to mediate activation when Ab is bound (5). FcγRIIB, the inhibitory receptor, does not pair with the common γ-chain, but rather has an intracellular ITIM sequence and mediates inhibitory signaling (9). The balance of positive and negative signals determines the outcome of the interaction of Ab-bound microbes with cells, because the threshold to trigger phagocytosis or other events is based on the ratio of activating/inhibitory receptor engagement (10). Because Ab isotypes have different affinities for the various FcγRs, they can trigger different effector functions based on their receptor binding specificity. It is critical to note that although there is a similar system in humans, mouse IgG isotypes and FcγRs are not synonymous with human ones; although the specificities of human IgG isotypes for the various human FcγRs are well characterized (11, 12), the mouse system is different and less well understood. Various studies in mice showed that IgG2a is the most promiscuous Ab, interacting with all FcγRs, whereas IgG1 is more selective and only binds to one activating receptor, FcγRII (13). Although much work has been done to study the interaction of the various Ab isotypes with the different FcγRs, major questions still remain. In this regard, three of the four mouse IgG subclasses, IgG1, IgG2a, and IgG2b, have been well characterized in terms of their affinity and specificity for the different FcγRs (14, 15); however, the data for IgG3 have been inconsistent, and most current reviews conclude that IgG3 interacts only very weakly with known FcγRs (13). Understanding the mouse FcγR system is important because mice continue to be the most commonly used animal system for immunological studies.

In 1981, Diamond and Yelton (16) proposed that a unique IgG3 receptor existed, based on the spontaneous J774 cell variant that specifically lost the ability to phagocytose sheep red blood cells coated in IgG3 but retained the ability to phagocytose via the other Ab isotypes. A subsequent study by Gavin et al. (17) showed that the known receptor FcγRI, which has high affinity for IgG2a, could also interact with IgG3. However, this study demonstrated only low-affinity binding of IgG3 to FcγRI-transfected cells. Additionally, in bone marrow–derived macrophages from FcγRI-deficient mice, phagocytosis of IgG3-coated particles failed compared with macrophages derived from wild-type mice in which they visualized internalization via IgG3. However, this study was not consistent with the original observation by Diamond and Yelton (16), in which phagocytosis by the variant cell line via other Ab isotypes, such as IgG2a, was unchanged, which would not be true if FcγRI was the receptor lost by the J774 variant. Moreover, the study by Gavin et al. (17) did not examine the role of IgG3 phagocytosis in terms of a microbe-specific Ab interaction with host cells during infection. The role of IgG3 in phagocytosis is not clear. In addition to these studies, there is other evidence strongly supporting the notion that IgG3 Abs engage a different receptor than other IgG subclasses: 1) IgG1 and IgG3

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Abbreviations used in this paper: CR, complement receptor; Lamp1, lysosomal-associated membrane protein 1.

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have very different protective efficacy in mice (18); 2) IgG1 is toxic, whereas IgG3 is nontoxic in mice with chronic cryptococcal infection and high serum Ag levels (19, 20); and 3) IgG3, but not the other IgG subclasses, mediates Ag–Ab complex enhancement of the Ab response in Fcγ-deficient mice (21).

In this study, we revisited the topic of IgG3-mediated phagocytosis by comparing the opsonic efficacy of IgG1 and IgG3 for Cryptococcus neoformans. Many mAbs specific for C. neoformans have been generated and used to study the effects of passive mAb treatment on cryptococcal infection (22–24). The in vitro system of C. neoformans and macrophage interactions is ideal to study receptor-mediated phagocytosis because in the absence of opsonin, phagocytosis of C. neoformans by macrophages is essentially zero, and yeast cells are easily identified and counted by microscopy (25–27). However, this system has some unique features that need to be taken into account, including the phenomenon of Ab-mediated phagocytosis proceeding through FcγRs and complement receptors (CRs) in the absence of complement. In this system, IgG can function as directly opsonizing Ab, where the Fc portions of Ab bound to the capsule of C. neoformans are recognized by FcγRs on phagocytic cells. In addition to Fc-mediated phagocytosis through FcγRs, CRs can mediate phagocytosis independently of complement through a mechanism whereby Ab binding to the capsule of C. neoformans causes a conformational change that allows capsular polysaccharide to directly interact with CD18, the common component of the dimeric receptors CR3 and CR4 (28). Because complement can also be an opsonin for C. neoformans, our experiments excluded all sources of complement to focus specifically on Ab-receptor interactions. Ab-mediated phagocytosis of C. neoformans is a system that has been extensively studied, and the relevant parameters are so well understood that C. neoformans phagocytosis by IgG1 mAb 18B7 has been subjected to mathematical modeling, producing a system whereby the efficiency of phagocytosis was accurately described by 11 differential equations (29). Using primary mouse macrophages and the 3E5 mAb generated and used to study the effects of passive mAb treatment on the in vitro isotype switching. Ascites was generated by injecting hybridoma cells into the peritoneal cavity of pristine BALB/c mice (National Cancer Institute, Frederick, MD), and harvesting the fluid was performed from ascites using a protein G column following the manufacturer’s instructions (Pierce, Rockford, IL) and then dialyzed in PBS and quantified by ELISA with an isotype-matched standard to determine concentration. Abs were aliquoted, stored in PBS at −20°C, and thawed once before use.

**Phagocytosis assays**

Phagocytosis assays were performed in 96-well plates containing primary peritoneal cells isolated 1 d prior to the experiment. In the case of CR- or FcγR-blocking experiments, Abs to CR3 and CR4 (CD11b, CD11b, and CD11c; BD Pharmingen, San Diego, CA) or the 2.4G2 Ab (BD Pharmingen) were allowed to bind for 30–60 min at 37°C. Then the IgG1 or IgG3 solution was added together with a C. neoformans suspension in RPMI 1640, for a final volume of 200 μL, with blocking Abs at 10 μg/mL, opsonizing IgG1 and IgG3 Abs at 10 μg/mL (unless otherwise noted), and C. neoformans at an E:T ratio of 1:1, with 1 × 10^7 C. neoformans yeast cells/well. Phagocytosis was allowed to proceed for 2 h at 37°C in 10% CO2. Cells were washed three times with PBS, fixed with methanol at −20°C for 30 min, washed again with PBS, and stained with Giemsa diluted 1:20 with sterile water. Cells were analyzed under an inverted microscope, counting three fields/well, with ≥100 cells/field. Macrophages with internalized C. neoformans were readily distinguishable from cells that had taken up C. neoformans or cells to which C. neoformans was attached on the outside, because of the visible vacuole containing engulfed C. neoformans. Experiments performed with Uvitex dye confirmed the accuracy of light microscopy for the determination of ingested cells (data not shown), whereby extracellular C. neoformans are distinguishable from intracellular C. neoformans by the exclusion of dye from C. neoformans that have been internalized by macrophages, as described previously (31). The percentage of phagocytosis was calculated as the number of macrophages containing one or more C. neoformans divided by the total number of macrophages visible in one field. Experiments were performed with each condition in triplicate and values were averaged, and t tests were used for statistical comparisons. Experiments were repeated on separate days as indicated in the figure legends.

**Immunofluorescence and microscopy**

Primary peritoneal cells were isolated as described above, grown on glass-bottomed plates (MatTek, Ashland, MA), and allowed to adhere overnight at 37°C in 10% CO2. For time-lapse images, phagocytosis assays were performed under the same conditions as above and allowed to proceed for 2 h, after which cells were washed three times and maintained in fresh media. Cells were then analyzed on a Zeiss microscope in a special cabinet that maintained conditions of 37°C in 10% CO2, and images were collected every 4 min for up to 24 h. Videos and montages were generated by capturing these images. For confocal images, these assays and fixation were carried out under the same conditions as above. Then cells were blocked with PBS + 1% BSA and stained with lysosomal-associated membrane protein 1 (LAMP1) Ab directly conjugated to FITC (BD Pharmingen) at a dilution of 1:100 for 1 h at 37°C; finally, cells were washed and mounted in 0.1 M propyl gallate (Sigma-Aldrich, St. Louis, MO) solution in 50% glycerol to minimize quenching. Cells were analyzed on a Zeiss microscope and compared with unstained cells at the same exposure time to account for autofluorescence, which was minimal.

**Radiolabeled ligand binding and Scatchard analysis**

Peritoneal macrophages were harvested from mice and resuspended in PBS in microcentrifuge tubes preblocked with BSA, in suspensions of 5 × 10^6 cells/ml for condition I or 3.5 × 10^6 cells/ml for condition II. Condition I used cells from FcγR−/− and Fcγ−/− mice, and condition II used cells from FcγR−/− and Fcγ/FcγRII−/− mice. For both experiments, IgG1 and IgG3 were radiolabeled with 188Re eluted from [188Re]ReCl₃⁺ generator (Oak Ridge National Laboratory, Oak Ridge, TN), as described previously (32). 188Re-labeled IgG1 and IgG3 were added to the cells at concentrations of 0.08–0.32 nM (equivalent to concentrations of 0.012–0.048 μg/mL). After
incubation for 1 h at 37°C (for condition I) or at 4°C (for condition II), the tubes were counted in a gamma counter; the cells were collected by centrifugation, and the pellets were counted again. Scatchard analysis was used to compute the mAb-binding constant ($K_a$) and binding sites per cell, as described previously (33).

Statistical analysis

The Student t test was used to compare the averages of the percentage of phagocytosis between experiments, with the Bonferroni correction for multiple comparisons.

Results

IgG1 and IgG3 show similar efficacy in phagocytosis

First we analyzed the opsonic efficacy of IgG1 and IgG3 in promoting phagocytosis of C. neoformans as a function of Ab concentration with wild-type C57BL/6 (FcR$^{+/+}$) macrophages. Although there was some variation from experiment to experiment, IgG1 and IgG3 mAbs were opsonic for C. neoformans was some variation from experiment to experiment, IgG1 and IgG3 as described previously (33).

To block FcRs were shown to be effective at inhibiting phagocytosis via these CRs (28). In these experiments, virtually no phagocytosis occurred in the absence of opsonin (30). An Ab concentration of 10 μg/ml was the lowest that provided optimal phagocytosis. A statistical analysis showed that there was no significant difference between the efficacy of IgG1 and IgG3; thus, this concentration was used throughout the rest of the experiments.

Contribution of FcγRs and CRs in Ab-mediated phagocytosis

To explore the roles of the different receptors in IgG1- and IgG3-mediated phagocytosis, we used both receptor-blocking conditions as well as macrophages deficient in FcγRs or CRs. In phagocytosis assays with FcγRI/RII/RIII macrophages, mAb 2.4G2 was used to block FcγRs. 2.4G2 is specific for FcγRII and -III and was originally used to clone and identify the first FcγRs (34). The CRs CR3 (CD18/CD11b) and CR4 (CD18/CD11c) were shown to be involved in Ab-mediated phagocytosis of C. neoformans, and Abs to CD18, CD11b, and CD11c were shown to be effective at inhibiting phagocytosis via these CRs (28). To block FcγRs or CRs, FcγRI/RII/RIII macrophages were preincubated with specific Abs. Under CR block, IgG1 phagocytosis was reduced to ~31% of the level of phagocytosis with no block and to ~8.5% under FcγR block (Fig. 2). With FcγR and CR blocked, IgG1 phagocytosis was similar to the level of the control with no opsonin. However, IgG3 had high levels of phagocytosis under all conditions and was apparently unaffected by the addition of FcγR block. The two conditions with CR block reduced phagocytosis via IgG3 by ~15–25% (Fig. 2). The difference between IgG1- and IgG3-mediated phagocytosis was not statistically significant under the condition with no block; however, IgG1-mediated phagocytosis under CR block was significantly lower compared with IgG1-mediated phagocytosis with no block or to IgG3-mediated phagocytosis under CR block. Although not indicated in Fig. 2, IgG1-mediated phagocytosis was also significantly reduced under conditions of Fc block and CR + Fc block.

Because the blocking experiments indicated that IgG3 was able to induce phagocytosis while the known opsonic receptors were blocked, we next analyzed the interaction of Ab with cells deficient for certain receptors. Mice deficient in the common $γ$-chain lack functional versions of all known activating FcγRs (Fcγ$^{−/−}$). IgG1-mediated phagocytosis of C. neoformans with Fcγ$^{−/−}$ cells compared with Fcγ$^{+/+}$ cells was significantly reduced (Fig. 3). If cells were preincubated with CR-blocking Abs, IgG1-mediated phagocytosis was essentially abrogated in these cells. However, IgG3-mediated phagocytosis still occurred at comparable levels in Fcγ$^{−/−}$ cells, which were not significantly different from wild-type levels (Fig. 3), indicating that this isotype was able to induce C. neoformans phagocytosis, even in the absence of all activating FcγRs and with blocked CR. Similarly, cells from mice deficient for the common $γ$-chain and the inhibitory receptor FcγRII (double knock-out FcγR/FcγRII$^{−/−}$) were able to efficiently phagocytose IgG3-coated C. neoformans, even in the presence of CR-blocking Abs, whereas IgG1-mediated phagocytosis failed (Fig. 3). Finally, phagocytosis was evaluated in mice deficient for CD18, the common component of CR3 and CR4, the receptors involved in binding to the C. neoformans capsule (28). In these cells, IgG3-mediated phagocytosis, alone and in the presence of Fc-block conditions, was essentially unchanged from wild-type levels (Fig. 3). However, IgG1-mediated phagocytosis was significantly reduced in the CD18$^{−/−}$ cells (55% of wild-type levels), and IgG1 phagocytosis failed when Fc block was present.

Additionally, we addressed the possibility that FcγRI is responsible for binding and phagocytosis via IgG3, as posited by other researchers (17). In cells from mice deficient for the $α$-chain of FcγRI (FcγRI$^{−/−}$), IgG3 still had high levels of phagocytosis, when CRs were blocked (Fig. 4), indicating that another receptor must be responsible for IgG3-mediated ingestion. Overall, the levels of IgG1- or IgG3-mediated phagocytosis in FcγRI$^{−/−}$ cells...
were not different from the levels in wild-type mice, when comparing the two cell types with or without CR block for each Ab. To confirm that there was a reduction of functioning FcγRI in these knockout mice, we also used IgG2a, a third isotype switch variant of mAb 3E5 (30). FcγRI is the high-affinity receptor for IgG2a and probably accounts for the majority of IgG2a recognition by cells (35). In phagocytosis assays, IgG2a and IgG1 were similarly effective in promoting C. neoformans uptake in FcR+/+ cells (Fig. 4). However, IgG2a was significantly less effective in promoting phagocytosis with FcγRII−/− cells, confirming that FcγRI is the main receptor responsible for IgG2a phagocytosis and that it was severely reduced in the knockout cells. However, IgG3 phagocytic efficacy was unaffected by the absence of FcγRI (Fig. 4).

To account for this complex set of interactions, we propose a model that posits the existence of an IgG3-specific receptor (Fig. 5). Here, the known activating FcγRs (FcγRI, FcγRII, and FcγRIV) and the CRs known to interact with Ab-coated C. neoformans (CR3 and CR4) are depicted, and their opsonic interactions with C. neoformans are indicated with solid green lines. No phagocytosis occurs in the absence of Ab or other opsonin. Phagocytosis of IgG1-coated C. neoformans is attributable to CR and FcγRII. IgG2a uses CR and mainly FcγRI, although the other FcγRs may also contribute, because IgG2a was shown to interact with all activating FcγRs (possible interactions indicated by dotted lines). For IgG1 and IgG2a, eliminating CRs and FcγRs abrogates their phagocytic function. However, IgG3-mediated phagocytosis still occurs in the absence of these receptors, and we propose that its function can be explained by the presence of some unknown receptor (X). For IgG3, CRs were shown to be involved, and although phagocytosis in the absence of FcγRs still occurs normally, we cannot say that FcγRs do not interact with IgG3 at all; hence, their interaction in terms of phagocytosis is questionable in our system (indicated by dotted black lines). However, in the absence of CRs and FcγRs, IgG3 is still an efficient opsonin, presumably through the X receptor.

Outcome of internalization of C. neoformans
To confirm that phagocytosis of C. neoformans via IgG3 leads to internalization and maturation of phagocytic compartments, we performed IgG3 phagocytosis assays with FcγRI−/− cells under CR block and then stained for LAMP1, an intracellular marker of the phagolysosomal compartment (Fig. 6A). C. neoformans opsonized with IgG3 in FcγRI−/− cells with CR blocked localized to LAMP1-positive compartments. For comparison, in FcγRI−/− cells with CR blocked, no phagocytosis of C. neoformans occurred via IgG1; therefore, LAMP1-positive compartments showed no internalized C. neoformans in any field examined (Fig. 6B).

To investigate the outcome of opsonization through the different receptors, we evaluated LAMP1 staining as a function of time after IgG1- and IgG3-mediated phagocytosis of C. neoformans in wild-type and knockout cells with CR blocked. Although there were some differences in the intensity and pattern of LAMP1 staining, overall, the vast majority of internalized C. neoformans localized to LAMP1-compartments following phagocytosis at 2, 4, and 6 h with IgG1 and IgG3, and we found no significant differences between experimental conditions when quantifying these results (data not shown). Additionally, we analyzed the cryptococcal load of phagocytic macrophages by averaging the number of intracellular C. neoformans in each macrophage under different conditions. The average numbers of C. neoformans per macrophage for each condition were as follows: 2.6 ± 0.8 for FcγRII–/– CR-blocked cells with IgG1, 1.6 ± 0.4 for FcγRII–/– CR-blocked cells with IgG3, and 2.3 ± 0.6 for FcγRI−/− CR-blocked cells with
IgG3. There was no significant difference among these numbers, suggesting that phagocytosis via the different receptors led to a comparable C. neoformans load.

By compiling light microscopy time-lapsed images taken over 24 h into videos, we were able to follow the interaction of macrophages and C. neoformans and compare differences in the effects of IgG1 or IgG3 opsonization. Previous work established that Ab-mediated phagocytosis of C. neoformans by macrophages can result in such events as intracellular cryptococcal replication and exocytosis, in which the macrophage expels internalized C. neoformans, leaving the macrophage and C. neoformans viable (36). To assess IgG1 interactions with FcγR, we used FcRγR–/– macrophages with CR blocked to eliminate the possibility of CR promoting phagocytosis; we found that internalized C. neoformans underwent intracellular replication and exocytosis events (Fig. 7A). When IgG3 was analyzed with FcγRI/– macrophages under CR block, eliminating the contribution of known FcγRs and CRs, we also observed successful phagocytosis followed by intracellular fungal replication and exocytosis events (Fig. 7B). Hence, by all parameters studied, IgG1- and IgG3-mediated opsonization of C. neoformans produced similar outcomes.

Scatchard plot shows specific binding of IgG3 to cells

To evaluate the possibility of an unknown receptor for IgG3, we examined the interaction of radiolabeled Ab with primary cells. Scatchard analysis revealed that IgG3, but not IgG1, bound significantly to FcγRII/– cells, consistent with the notion that a unique cellular receptor for IgG3 exists (Table I). The affinity of IgG3 for FcγRII/– cells was 1.5 × 10^{9} M^{-1}, whereas the number of binding sites was determined to be 6 × 10^{4} per cell. When IgG3 was added to FcRγR+ cells, binding was similar (2.0 × 10^{9} M^{-1}), as were the determined number of binding sites (7 × 10^{4} per cell). There was low binding of IgG1 to FcRγR+ macrophages, consistent with previous data reported on IgG1 interacting with low-affinity receptors (14). Similarly, IgG1 binding to FcγRII/– cells was nonspecific; linear regression analysis showed that the slope was not significantly different from zero, consistent with and indicative of the absence of a receptor (Table I). The first Scatchard analysis was performed at 37˚C to mimic the in vivo conditions of Ab–cell interactions. To rule out the possibility that Ab binding could cause turnover of cellular receptors, we repeated this analysis at 4˚C, using double-knockout cells deficient for the inhibitory FcγR as well as the Fcγ chain (Fcγ/FcγRII–/–) and obtained similar results. Here, the affinity of IgG3 for Fcγ/FcγRII–/– cells was 2.3 × 10^{9} M^{-1}, and the number of binding sites was determined to be 1.3 × 10^{5} per cell. Once again, IgG1 showed no significant interaction with Fcγ/FcγRII–/– cells.
Discussion

We revisited the issue of IgG3 engagement of FcγRs by looking at its functional role in phagocytosis using several new tools that have become available since this issue was last investigated (16, 17). Specifically, the availability of mice with selective deficiencies of FcγR expression in combination with a relatively clean assay of phagocytosis in the form of *C. neoformans* interaction with macrophages, new serological reagents to block other opsonic receptors, and a well-characterized IgG1 and IgG3 isotype switch pair specific for *C. neoformans* provided a new system to reevaluate the old question of whether IgG3 engages a different receptor (16).

The IgG3 isotype is the least studied isotype among murine IgG subclasses because it is relatively rare among mAbs and has a propensity to behave as a cryoglobulin (37). IgG3 has the remarkable capacity to self-aggregate after binding Ag, thus providing for a mechanism for increased avidity (38). Indeed, IgG3 is the major isotype produced against polysaccharide Ags in mice, yet its role in the immune response is not fully understood.

Our findings show that IgG3 is highly effective at promoting *C. neoformans* phagocytosis in mouse cells deficient for known FcγRs with CRs blocked and in cells deficient for CD18 with FcγRs blocked, whereas IgG1 is completely ineffective in these conditions. This observation necessarily implies that IgG3 is promoting phagocytosis through a different type of receptor than the classic FcγR receptors engaged by IgG subclasses. Furthermore, this phagocytosis seems to be functionally comparable to that observed with the other FcγRs, because the internalized *C. neoformans* localize to mature, LAMP1-positive...
phagolysosomal compartments and exhibit similar behavior with regard to the intracellular replication of internalized *C. neoformans* and exocytosis events.

In revisiting the subject of IgG3 phagocytosis, we considered the explanation proposed by Gavin et al. (17), i.e., that IgG3 can promote phagocytosis through FcγRII. However, our observations that macrophages from FcγRII−/− mice efficiently phagocytosed *C. neoformans* in conditions of CR blockade strongly argued that FcγRII is not the receptor responsible for promoting IgG3 phagocytosis. In fact, this conclusion is in agreement with the observations that led to the original proposal that IgG3 used a different receptor. When Diamond and Yelton (16) concluded in 1981 that IgG3 bound to a unique cellular receptor, they based their conclusion on the observation that a spontaneous variant of the macrophage-like cell line J774 had lost the ability to phagocytose particles coated in IgG3. However, the J774 cell variant used in that study still retained the capacity for phagocytosis of IgG2a, as well as the other IgG isotypes. Presumably, if IgG2a phagocytosis was normal, then the function of FcγRII was also normal, and the lost activity of IgG3 must be due to some unidentified receptor.

If IgG3 does promote phagocytosis through a nonclassic FcγR type, it must bind to macrophages that lack the known FcγRs. To explore this possibility, we used a radiolabeled ligand-binding approach to determine if IgG3 could bind Fcγγ2a or Fcγ/Fcγ RIIC/− primary cells. Macrophages deficient for γ-chain expression lack functional FcγRI, FcγRIII, and FcγRIIV receptors, and the double-knockout cells also lack the inhibitory receptor FcγRII. The approach taken was Scatchard analysis for IgG1 and IgG3 and comparing macrophages from Fcγ1/−/− mice with Fcγγ2a (condition I) or Fcγ/FcγRIIC/−/− mice (condition II). Additionally, the first experiment was performed at 37˚C (condition I) to mirror relevant Ab-receptor interactions in vivo, and the second experiment was performed at 4˚C (condition II) to reduce the level of receptor internalization and turnover after Ab binding. As expected, IgG1 showed no specific binding to macrophages from Fcγγ2a or Fcγ/FcγRIIC/−/− mice. Similarly, IgG1 demonstrated binding to Fcγ1/−/− (Kd = 2.2 × 10^8 M^-1 for condition I and 1.6 × 10^9 M^-1 for condition II), consistent with reports that this isotype has low affinity for FcγR (14). In contrast, IgG3 showed higher-affinity binding to macrophages from Fcγ1/−/−, Fcγγ2a, and Fcγ/ FcγRIIC/−/− mice. The affinity of IgG3 for the new putative receptor in macrophages and the receptor number on cells from Fcγγ2a mice (1.5 × 10^9 M^-1 and 8 × 10^9 binding sites/cell) or Fcγ/FcγRIIC/−/− mice (2.3 × 10^8 M^-1 and 1.3 × 10^9 binding sites/cell) were comparable to the range of affinities and receptor numbers reported for the other IgG-FcγR interaction and other FcγRs, respectively (14, 39). Of interest, the affinity of IgG3 was lower in condition II (4˚C) compared with condition I (37˚C), which is consistent with the lower temperature slightly decreasing the affinity of Ab for receptor. Also supporting this difference is the slightly higher number of IgG3-binding sites in condition II (4˚C), which indicated that the lower temperature decreased the level of receptor turnover, hence, slightly increasing the number of available receptors per cell. Taken together, these data indicate the presence of a high-affinity receptor for IgG3 on the surface of mouse cells that is different from the other known FcγRs.

Additionally, the phagocytosis data provide insights into the proportion of involvement from FcγR or CR in IgG1-mediated phagocytosis. With CR blocked, IgG1 phagocytosis decreased by about one third in Fcγ1/−/− cells, and a similar decrease was seen with IgG1 in CD18−/− cells. In Fcγγ2a−/− cells, IgG1 phagocytosis decreased by about two thirds relative to that observed with FcγRIκκ macrophages, indicating that FcγRs seem to account for the majority of phagocytosis, and CRs account for the remainder.

Given our results implying that IgG3 promoted phagocytosis through a nonclassic FcγR, we evaluated the functional outcome of IgG3-mediated opsonization of *C. neoformans*. *C. neoformans* is a facultative intracellular pathogen that replicates intracellularly in mature phagosomes after IgG1- or C-mediated phagocytosis (40, 41). IgG3-mediated phagocytosis of *C. neoformans* by FcγR−/− cells with CR blocked resulted in fungal cell ingestion into a membrane-bound mature phagosome that was decorated by LAMP1. Intracellular residence in Fcγγ2a−/− macrophages was followed by fungal replication and occasional phagosome extrusion in a manner that was qualitatively similar to that observed for IgG1-mediated phagocytosis in Fcγ1/−/− macrophages with CR blocked (36). Hence, in the *C. neoformans* phagocytosis system, phagocytosis via IgG1 using classic FcγRs or via IgG3 using the nonclassic, putative IgG3 receptor produced quantitatively and qualitatively similar outcomes for the fungal–macrophage interaction.

In summary, we provide strong evidence that murine IgG3 interacts with a cellular receptor that is structurally different from the classic FcγRs in that it lacks the γ-chain. Our results are consistent with the original proposal by Diamond and Yelton (16) for a distinct IgG3 receptor and are supported by several studies showing major differences in the biological effects of IgG3 versus the other IgG subclasses (18–21, 30). Understanding Ab-receptor interactions is essential to fully grasp the role of isotype in Ab responses induced during infection or to discover the mechanism of Ab-mediated effects during passive mAb therapy. Given that different isotypes were shown to have very different roles when administered in vivo (42), and given that passive Ab treatment is a growing field that is dependent on research and development occurring in the mouse model and ultimately leading to human therapies (7, 8, 43), it is essential to understand mouse IgG-receptor interactions for all isotypes. This research highlights the additional information we need to learn about this system. The results presented here suggest that future studies focus on the molecular and structural characterization of this receptor.

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**Disclosures**

The authors have no financial conflicts of interest.

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