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Immunotherapy Targeting Inhibitory Fcγ Receptor IIB (CD32b) in the Mouse Is Limited by Monoclonal Antibody Consumption and Receptor Internalization

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Genetic deficiency of the inhibitory Fc receptor, FcγRIIB (CD32b), has been shown to augment the activity of activatory FcγR and promote mAb immunotherapy. To investigate whether mAbs capable of blocking FcγRIIB have similar capacity, we recently generated a panel of specific anti-mouse FcγRIIB mAbs that do not cross-react with other FcRs, allowing us to study the potential of FcγRIIB as a therapeutic target. Previous work revealed a number of these mAbs capable of eliciting programmed cell death of targets, and in the present study we demonstrated their ability to promote target cell phagocytosis. However, in a variety of murine tumor models, anti-FcγRIIB mAbs demonstrated limited therapeutic activity despite optimized treatment regimens. Unexpectedly, we observed that the anti-FcγRIIB mAbs are rapidly and extensively consumed in vivo, both by the tumor and host cells, including B cells, leading to a precipitous loss from the circulation. Closer analysis revealed that the anti-FcγRIIB mAbs become extensively internalized from the cell surface within 24 h in vivo, likely explaining their suboptimal efficacy. Subsequent studies revealed that anti-FcγRIIB mAb immunotherapy was effective when used against FcγRIIB+ tumors in FcγRIIB−/− recipients, indicating that consumption of the mAb by nontumor cells is the primary limitation of these reagents. Importantly, similar rates of internalization were not seen on human target cells, at least in vitro. These studies further highlight the need to determine the propensity of mAb therapeutics to internalize target receptors and also identify potential key differences between human and mouse cells in this respect. The Journal of Immunology, 2013, 191: 000–000.

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heerapeutic Abs such as rituximab, trastuzumab, and bev-acizumab are now established treatments for various cancers (1, 2). A major component of their therapeutic efficacy is thought to derive from their interaction with FcγRs (3). FcγRs are expressed on a wide range of immune cells such as macrophages, neutrophils, and NK cells, as well as B cells and dendritic cells (DCs) (4, 5). Both human and mouse FcγRs fall into two subgroups: activatory and inhibitory. Signaling through the activatory FcγR (FcγRI, III, and IV in the mouse) occurs via an ITAM-containing cytoplasmic tail or association with a common ITAM-containing γ-chain. Conversely, inhibitory signaling is afforded by the ITIM-containing tail of FcγRIIB/CD32b. With the exception of FcγRI (and IgG2a binding to FcγRIIV), the FcγRs have relatively low affinity for monomeric IgG (K_d of ~10^6 M−1) and so are unlikely to engage IgG in free solution.

FcγR function varies with the cell type on which they are expressed. Triggering activatory FcγRs on effector cells, such as monocytes or macrophages, results in the release of a spectrum of inflammatory immune modulators, such as cytokines. Conversely, triggering of FcγRIIB on B cells by IgG-containing immune complexes (ICs) serves to inhibit signaling through the BCR, reducing proliferation, Ab production, and potentially delivering apoptotic signals to the cells (reviewed in Ref. 4). More recently it has been shown that FcγRIIB is also expressed on plasma cells (and myeloma cells). In such cases, engagement can also induce apoptosis with potentially important implications for controlling long-lived plasma cells and shaping the Ab repertoire (6). The coexpression of both inhibitory and activatory FcγR by cells provides a mechanism of fine control for cellular activation, where engagement of the FcγRIIB counterbalances activatory FcγR effector function.

Within the field of Ab-based immunotherapy, growing evidence indicates that FcγRs on innate effector cells are critical for controlling therapeutic efficacy (7–10). In mouse models the therapeutic activity of a range of anti-cancer mAbs correlates with their ability to bind to activatory FcγR (8, 11–16), and deletion of the FcγR γ-chain abrogates therapeutic activity of most anti-cancer mAbs (7). In contrast, in their seminal paper, Clynes et al. (7)
demonstrated that deleting FcγRIIB enhances the therapeutic activity of a number of different therapeutic mAbs, including 4D5 and trastuzumab (17), further alluding to the balance between FcγRIIB and activatory FcγR being critical for mAb-induced effector function.

Evidence for similar activity in humans has been more difficult to obtain and is largely derived from genetic studies, taking advantage of the fact that certain FcγRs have polymorphisms that affect their affinity for various IgG classes. Polymorphisms that increase the interaction between IgG and FcγRIIA or FcγRIIIA have been shown to be associated with improved therapeutic efficacy (18–21). Similarly, defucosylated IgG, which binds with a marked increased affinity to the activatory FcγRIIIA, shows ~50-fold increase in Ab-dependent cellular cytotoxicity (ADCC) activity, which is expected to deliver significant improvement in clinical efficacy. In both mice and humans, polymorphisms that result in reduced expression or activity of the inhibitory FcγRIIB are associated with increased susceptibility to autoimmune disease (22–28).

For these reasons, FcγRIIB has long been seen as a potential target for immunotherapy, both as a means of augmenting effector cell activation and as a way of transducing death signals to target cells. Unfortunately, suitable mAb reagents capable of specifically and exclusively binding mouse FcγRIIB have largely been lacking, making specific in vivo manipulation of FcγRIIB impossible. Although human FcγRIIB-specific mAbs have recently been produced (29–31), these have only been tested in xenograft systems to date, making their true therapeutic potential unclear.

Following an extensive immunization protocol using FcγRIIB−/− mice, we recently produced unique mAbs that specifically recognize FcγRIIB, block immune complex binding, and are capable of regulating its activity. In particular, the agonistic mAbs (AT130-2 and AT130-5) were able to phosphorylate the receptor, inhibit BCR-induced Ca2+ flux, and trigger programmed cell death (PCD) in B lymphoma cells (32). In contrast, the antagonistic mAbs (AT128 and AT128 m2a, along with the FcγRIIB/IIIa dual-specific mAb 2.4G2) evoked relatively little PCD and augmented Ca2+ responses. Having previously characterized these mAbs (32), in this study we addressed the potential efficacy of these reagents in vivo in fully syngeneic model systems. Using a variety of these models we have revealed a hitherto unexpected limitation of these reagents that is associated with their propensity to become modulated from the cell surface.

Materials and Methods

Animals and cells

Mice were bred and maintained in local facilities. Human (h) CD20 transgenic (Tg), γ-chain−/−, and FcγRIIB−/− mice have been described previously (33, 34), as have the FcγRIIB−/−CD19Cre+ mice (34–36). Genotypes were confirmed by PCR and/or flow cytometry. Ten- to 12-wk-old female BALB/c and CBA/H mice were supplied by Harlan UK (Blackthorn, Oxon, U.K.) and maintained in local animal facilities. BCL1 (37) and A31 (38) mouse B lymphoma models and the ST-33 myeloma (gift from Dr. N. Saveleva) were maintained in vivo by passage in BALB/c, CBA, and C57BL/6 KA mice, respectively. Animal experiments were cleared through the local ethical committee and performed under Home Office licenses PPL30/2450 and 30/2451. naïve BCL1 is a variant of BCL1 that arose from the wild-type BCL1, and is capable of growth both in culture and also in vivo (39). Cell culture was performed in supplemented RPMI 1640 (RPMI 1640 containing 2-ME [50 μM], glutamine [2 mM], pyruvate [1 mM], penicillin and streptomycin [100 IU/ml], fungizone [2 mg/ml], and 20% FCS [Myoclone]) (Life Technologies BRL, Paisley, U.K.). Mouse splenic B cells were purified by negative selection using MACS B cell isolation kits (Miltenyi Biotec) and cultured in supplemented RPMI 1640. Human cell lines (Daudi, Raji) were obtained from the European Cell Culture Collection (Porton Down, U.K.) and were maintained in antibiotic-free RPMI 1640 containing glutamine (2 mM), pyruvate (1 mM), and 10% FCS (Myoclone) (Life Technologies). Normal human peripheral B cells were purified by negative selection using MACS B cell isolation kits (Miltenyi Biotec).

Generation of bone marrow–derived macrophages

Murine bone marrow–derived macrophages (BMMs) were generated from cells isolated from the bone marrow of the femur and tibia of mice. Cells were cultured in RPMI 1640 (Life Technologies Invitrogen, Paisley, U.K.) enriched with 10% FCS, 2 mM glutamine and 1 mM pyruvate, penicillin and streptomycin (each at 100 μg/ml), and 20% L929–conditioned medium (containing M-CSF). Cells were cultured at 37°C, 5% CO2 for 10–14 d prior to use. Macrophage differentiation was routinely confirmed by morphological examination and/or flow cytometry for CD11b expression.

Abs and reagents

mAbs were typically produced from the culture supernatant of hybridoma cells or stably transfected Chinese hamster ovary–K1 (AT128 m2a). IgG was purified on protein A with purity assessed by electrophoresis (Beckman EP system; Beckman Coulter) and lack of aggregation was confirmed by HPLC. F(ab')2 fragments were produced as described previously (40). The anti-mouse FcγRIIB mAbs were raised using conventional hybridoma technology and were recently reported (32). AT130-2 and AT128 m2a are of IgG1 isotype, whereas AT128-5 and AT1282 are of IgG1 isotype. The anti-human FcγRIIB mAbs AT10 (41), IV3 (42), and KB61 (43) were previously described. Rituximab was gifted by Southampton General Hospital oncology pharmacy. Rit m2a was produced as described previously (33). The anti-mouse CD20 mAb 18B2 m2a was made by combining Vh and Vλ regions of the 18B12 IgG1 hybridoma with mouse γ chain and κ constant regions, respectively, and expressing the two polypeptide chains in Chinese hamster ovary cells (44). Anti-idiotype mAbs MC106A5 (BCL1) (45) and Mc39/16 (A31) (46) were generated in-house as previously described.

Flow cytometry

Fluorescently conjugated mAbs were purchased from BD Biosciences, AbD Serotec, or made in-house. Flow cytometry was as described previously (46) with data analyzed with CellQuest Pro or FACS DIVA (both BD Biosciences). To assay anti-FcγRIIB mAb (mouse IgG2a subclass) concentrations, sera were incubated with naïve BCL1 lymphoma cells (FcγRIIB+ and cell-bound anti-FcγRIIB mAb was detected using PE-labeled goat anti-mouse Fc (Jackson ImmunoResearch Laboratories) with reference to a standard curve obtained with known concentrations of anti-FcγRIIB mAbs. To determine surface expression of FcγRIIB after mAb treatment in vivo or in vitro, available Ag was saturated with excess anti-FcγRIIB mAb (10 μg/ml) and cells were washed and then surface anti-FcγRIIB detected using PE-conjugated goat anti-mouse Fc (Jackson ImmunoResearch Laboratories). Alternatively, to detect pre-existing surface accessible mAbs, cells were washed and incubated with the PE-conjugated goat anti-mouse Fc alone before analysis. B cells or macrophages were then identified with allophycocyanin-labeled anti-mouse CD19 and CD11b. Human cells were monitored in the same manner using mouse mAb and anti-mouse secondary detection reagents.

Measurement of anti-FcγRIIB mAb levels in serum

Anti-FcγRIIB mAb serum levels were monitored using a sandwich ELISA. Briefly, 96-well Immuno Maxisorb plates (Fisher Scientific, Leicester, U.K.) were coated with mouse anti-rat CD4 (OX68) F(ab')2 fragments (5 μg/ml) in ELISA coating buffer (15 mM Na2CO3, 28.5 mM NaHCO3, at pH 9.6) and incubated for 1 h at 37°C, then at 4°C overnight, followed by addition of FcγRIIB-C4D fusion protein diluted 1:2 with 1% BSA and incubated for 1 h. After 1 h the ELISA plate was washed with PBS/Tween 20 and the diluted sample was added to the wells for 90 min. The ELISA plate was then washed and goat anti-mouse Fc-y-HRP (Sigma-Aldrich, Dorset, U.K.) was added for a further 90 min before further washing and detection with o-phenylenediamine. The reaction was halted with 2.5 M H2SO4 and absorbance detected at 495 nm on the Dynatech MR400 plate reader, with the serum concentration of mAb calculated against a standard curve of known concentrations of anti-FcγRIIB mAb.

In vivo internalization assay

Fresh BCL1 tumor cells (2 × 107) were injected into female 8- to 12-wk-old wild-type (WT) or γ-chain−/− BALB/c mice i.v. on day 0. Mice received 0.5 mg mAb (anti-FcγRIIB or anti-idiotype) 4 d after tumor inoculation. Forty-eight hours later mice were sacrificed and spleens were
removed and assessed by flow cytometry for surface expression of the receptor along with surface-accessible mAbs as described above.

In vitro internalization assay and Alexa 488 labeling

mAbs were labeled with Alexa 488 (Invitrogen) according to the manufacturer’s instructions. To determine internalization, a quenching assay was performed as described previously (47). In brief, cell samples (2.4 × 10^6 cells/well) were incubated with Alexa 488–labeled mAbs (5 μg/ml for 2, 6, or 24 h at 37°C, washed, resuspended, and incubated at 4°C for 30 min in the presence or absence of anti-Alexa 488 quenching Ab (Invitrogen). Samples were then assessed by flow cytometry as described above. Only mAb that is remaining on the cell surface is accessible for quenching by anti-Alexa 488. Therefore, the results are expressed as percentage surface-accessible anti-FcγRIIB, which is inversely proportional to the amount of mAb internalized.

RT-PCR for FcγRIIB isoforms

Purified B cells or BMDMs were generated as indicated above and total RNA was isolated before conversion to cDNA SuperScript II first-strand synthesis system for RT-PCR (Invitrogen). PCR was performed using appropriate dilutions of cDNA and primers specific for murine FcγRIIB or murine β-actin as a control. Primers used were as follows: FcγRIIB, forward, 5′-CCC AAG TTC AGC AGG TCT TTA CGA GTA TTG-3′, reverse, 5′-TGC TTT AGA AGT GAG TAG GTG ATC GTA TCC-3′.

Phagocytosis assay

BMDMs were produced as detailed above and the phagocytosis assays were performed largely as detailed previously (47). Briefly, on the day of the assay, macrophages were harvested using trypsin/EDTA (Invitrogen), resuspended in RPMI 1640, and plated into a 96-well tissue culture plate (5 × 10^4 cells/well) and incubated for 2–4 h at 37°C. Target B cells were isolated from hCD20 Tg mice, labeled with 5 μM CFSE, washed, and then incubated with anti-FcγRIIB or anti-CD20 mAb for 30 min at room temperature. The B cells were then added to the BMDMs at a ratio of 5:1, the plates were incubated for 1 h at 37°C, and then allophycocyanin-anti-F480 Ab (AbD Serotec) was added to distinguish the macrophages. Cells were harvested and analyzed by flow cytometry. The percentage of cells that stained double positive for CFSE and allophycocyanin-anti-F480, which includes macrophages with both surface-bound and internalized B cells, was determined as a measure of phagocytic potential. Confirmation of phagocytosis was routinely provided by confocal microscopy (47).

Immunotherapy

Fresh murine syngeneic B lymphoma cells (1 × 10^6), either BCL1 or A31, were injected i.v. into cohorts of female 8- to 12-wk-old BALB/c or CBA/H mice, respectively (day 0). Each mouse (24 mice/group) received 0.5 mg of either an irrelevant isotype control (WT17) or anti-FcγRIIB mAb (AT130-2 or AT128 m2a) on day 3. On day 4, mice received 125 μg anti-idiotype mAb for 2, 6, or 24 h at 37°C, washed, resuspended, and incubated at 4°C for 30 min in the presence of or absence of anti-Alexa 488 quenching Ab (Invitrogen). Samples were then assessed by flow cytometry as described above. Only mAb that is remaining on the cell surface is accessible for quenching by anti-Alexa 488 and represents anti-FcγRIIB, which is inversely proportional to the amount of mAb internalized.

In xenograft models, Clynes et al. (7) showed that FcγRIIB−/− mice demonstrate an enhanced capacity to reject tumor cells following mAb immunotherapy. To confirm these findings using a syngeneic mouse tumor model, we compared anti-idiotype immunotherapy of the BCL1 tumor in WT and FcγRIIB−/− mice. In both WT and FcγRIIB−/− mice, anti-idiotype mAb improved survival in comparison with the nontreated mice (p < 0.01 for both). Nevertheless, in the FcγRIIB−/− mice there was a significant increase in survival compared with the same treatment in WT mice (p < 0.05) (Fig. 1).

**Results**

**Augmented immunotherapy in FcγRIIB−/− mice**

In xenograft models, Clynes et al. (7) showed that FcγRIIB−/− mice demonstrate an enhanced capacity to reject tumor cells following mAb immunotherapy. To confirm these findings using a syngeneic mouse tumor model, we compared anti-idiotype immunotherapy of the BCL1 tumor in WT and FcγRIIB−/− mice. In both WT and FcγRIIB−/− mice, anti-idiotype mAb improved survival in comparison with the nontreated mice (p < 0.01 for both). Nevertheless, in the FcγRIIB−/− mice there was a significant increase in survival compared with the same treatment in WT mice (p < 0.05) (Fig. 1).

**FcγRIIB mAbs elicit phagocytosis**

We next examined the efficacy of our panel of recently developed anti-FcγRIIB mAbs in a variety of mouse models of immunotherapy. We previously demonstrated that these mAbs were specific for FcγRIIB and able to manipulate FcγRIIB signaling in ways potentially leading to PCD of target cells (32). In addition to direct PCD, these mAbs also have the capacity to engender ADCC or phagocytosis of target cells. To investigate the ability of our anti-FcγRIIB mAbs to elicit phagocytosis, we generated BMDMs, which express all four FcγRs, and performed in vitro phagocytosis assays. Using CD20 Tg B cells as targets we were able to demonstrate that AT130-2 and AT130-5 were able to elicit phagocytosis but to a lesser extent than anti-CD20 mAbs, with AT128 m2a showing relatively little efficacy (Fig. 2A), probably due to its lower level of binding compared with AT130-2 (32). As expected, phagocytosis was fully dependent on the expression of activatory FcγR (ablated with γ-chain−/− macrophages) and was augmented by the loss of FcγRIIB on the macrophages (Supplemental Fig. 1). Interestingly, however, phagocytosis was not dependent on the isotype of the anti-FcγRIIB mAbs, with both IgG2a (AT130-2) and IgG1 (AT130-5) giving equivalent levels (Fig. 2A). Furthermore, blockade of FcγRIIB on the macrophages with AT130-2 or AT130-5 also resulted in augmentation of phagocytosis of anti-CD20 opsonized B cells, with AT128 m2a displaying a less robust effect (Fig. 2B and data not shown).

**FcγRIIB mAbs do not elicit significant immunotherapy in syngeneic mouse models**

We next examined the efficacy of our panel of recently developed anti-FcγRIIB mAbs in a variety of mouse models of immuno-

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**Statistical analysis**

To compare differences between experimental groups in vitro, two-tailed t test analysis was performed. To assess survival differences between experimental groups in vivo, Kaplan–Meier curves were produced and analyzed by log-rank testing. ANOVA analysis was used to determine differences between serum mAb levels. Statistical analysis was performed using GraphPad Prism software (version 5 for Windows).
therapy. Given our previous results demonstrating potent antitumor activities in vitro (32), we investigated the potential of our anti-FcRIIB mAbs alone did not result in any substantial improvement in survival in lymphoma (BCL1 or A31). We speculated that the tumor models used might have some additional regulatory mechanism able to counteract the anti-FcRIIB mAbs and so to determine whether the same phenomenon was occurring we investigated lymphoma cells as well as normal B-cells and BMDMs for their ability to internalize FcRIIB after treatment with AT130-2. Using both a conventional cell surface binding assay (Fig. 4B) and an Alexa 488-quenching assay (47) (Fig. 4C) to measure internalization, we were able to show cell surface modulation in each case. Similar results were seen with AT130-5 (data not shown). In the case of rituximab and CD20, the loss of mAbs occurred as a result of receptor internalization upon mAb engagement (47), and so to confirm this we performed experiments revealing that anti-FcRIIB-2 isoform on human cells. None of the three anti-human FcRIIB mAbs were modulated from either human cell lines or purified B-cells (Fig. 4D), whereas IgM modulated rapidly as expected.

One consequence of Rit m2a internalization is a comparatively rapid loss of mAbs from the serum of treated mice. Therefore, we assessed the serum levels of anti-FcRIIB mAbs from Fig. 4A. These experiments revealed that anti-FcRIIB mAbs were lost rapidly from the sera in comparison with the anti-idiotypic mAb, which was maintained at a much higher level (Fig. 5A). Notably, the BCR, which the anti-idiotypic recognizes, is expressed at a higher level on BCL1 cells than FcRIIB (Supplemental Fig. 3). FcRIIB expression was well maintained in each case (Supplemental Fig. 2). This is in contrast to the anti-mouse CD20 mAb (18B12), which was therapeutic in the BCL1 model (p < 0.001) (Fig. 3A). Furthermore, the anti-FcRIIB mAbs did not augment the anti-idiotypic immunotherapy in the BCL1 model (Fig. 3D), previously shown by us to be enhanced in the FcRIIB−/− mice (Fig. 1D). We speculated that the tumor models used might have some additional regulatory mechanism able to counteract the anti-FcRIIB mAbs and so examined the ability of the anti-FcRIIB mAbs to deplete normal B cells in vivo using the hCD20 Tg mouse model. A 0.5-mg dose of the anti-FcRIIB mAbs was unable to deplete any substantial depletion of peripheral B cells (Fig. 3E), splenic B cells, macrophages, or DCs, even when the treatment was repeated for three times (1.5 mg total) (Fig. 3F). In contrast, a single 250-µg dose of the anti-CD20 mAb Rit m2a resulted in profound depletion of peripheral B cells for >40 d (Fig. 3E) and substantial depletion of B cells in the spleen and lymph nodes (47).

**FIGURE 2.** Anti-FcRIIB mAbs elicit phagocytosis and can augment phagocytosis of opsonized target cells. (A) Purified hCD20 Tg B cells were CFSE labeled and then opsonized with 10 µg/ml anti-CD20 or anti-FcRIIB mAbs. These were then incubated with BMDMs at a 5:1 ratio for 1 h at 37°C. Cells were then harvested, stained with allophycocyanin-F480, washed once, and analyzed by flow cytometry with the proportion of CFSE/allophycocyanin double-positive macrophages used as a measure of phagocytosis. (B) BMDMs were pretreated for 1 h at 37°C with PBS, isotype control (WR17), or AT130-5 (10 µg/ml). These cells were then incubated for 1 h with CFSE-labeled CD20 Tg B cells preopsonized with no mAbs, rituximab (Ritux), or Rit m2a. Cells were harvested and analyzed as in (A). *p < 0.05, **p < 0.001.
Anti-FcγRIIB mAbs do not elicit robust responses in mouse models of immunotherapy. WT BALB/c (A) or CBA (B) mice were inoculated i.v. with $1 \times 10^5$ BCL$_1$ or A31 cells, respectively, on day 0. Mice were then treated with 0.5 mg AT130-2, AT128 m2a, or WR17 (irrelevant isotype control) on day 3. Concurrently, mice inoculated with BCL$_1$ cells were treated with 125 μg 18B12 m2a on days 1–4, and mice inoculated with A31 were treated with 125 μg anti-idiotype (Mc39/16) on days 1–4. (C) WT C57BL/6 KA mice were inoculated with $2 \times 10^5$ ST-33 cells on day 0. Mice were then treated with 0.5 mg AT130-2, AT128 m2a, or WR17 (irrelevant isotype control) on day 7. In each case AT130-2 and AT128 m2a did not increase survival of tumor-inoculated mice. 18B12 did improve survival of BCL$_1$-inoculated mice ($p < 0.05$). (D) WT BALB/c mice were inoculated with $1 \times 10^5$ BCL$_1$ as in (A) and treated with 0.5 mg AT130-2, AT128 m2a, or WR17 (irrelevant control) on day 3, followed by 125 μg anti-idiotype (Mc106A5) on days 4–7. In this instance, AT130-2 and AT128 m2a did not increase survival either alone or in combination with anti-idiotype immunotherapy. (E) WT C57BL/6 mice received 0.5 mg AT130-2 or irrelevant control (WR17) i.v. weekly for 4 wk. Circulating B cells were determined and expressed as a percentage of pretreatment B cell numbers. For comparison the level of B cell depletion is shown when a single 250-μg dose of an isotype control version of rituximab (Rit m2a) is used in congenic CD20 Tg mice. (F) WT BALB/c mice received 0.5 mg AT130-2 i.v. daily for 1, 2, or 3 d and the spleens were excised on days 2, 3, or 4, respectively. Splenocytes were stained for B cells (CD19 and B220), macrophages (F480 and CD11b), or DCs (CD11c and MHC class II) and analyzed by flow cytometry. Data are representative of at least three similar experiments, where $n = 4–5$ animals/group. Error is expressed as ± SEM.

**FIGURE 3.** Anti-FcγRIIB mAbs do not elicit robust responses in mouse models of immunotherapy. WT BALB/c (A) or CBA (B) mice were inoculated i.v. with $1 \times 10^5$ BCL$_1$ or A31 cells, respectively, on day 0. Mice were then treated with 0.5 mg AT130-2, AT128 m2a, or WR17 (irrelevant isotype control) on day 3. Concurrently, mice inoculated with BCL$_1$ cells were treated with 125 μg 18B12 m2a on days 1–4, and mice inoculated with A31 were treated with 125 μg anti-idiotype (Mc39/16) on days 1–4. (C) WT C57BL/6 KA mice were inoculated with $2 \times 10^5$ ST-33 cells on day 0. Mice were then treated with 0.5 mg AT130-2, AT128 m2a, or WR17 (irrelevant isotype control) on day 7. In each case AT130-2 and AT128 m2a did not increase survival of tumor-inoculated mice. 18B12 did improve survival of BCL$_1$-inoculated mice ($p < 0.05$). (D) WT BALB/c mice were inoculated with $1 \times 10^5$ BCL$_1$ as in (A) and treated with 0.5 mg AT130-2, AT128 m2a, or WR17 (irrelevant control) on day 3, followed by 125 μg anti-idiotype (Mc106A5) on days 4–7. In this instance, AT130-2 and AT128 m2a did not increase survival either alone or in combination with anti-idiotype immunotherapy. (E) WT C57BL/6 mice received 0.5 mg AT130-2 or irrelevant control (WR17) i.v. weekly for 4 wk. Circulating B cells were determined and expressed as a percentage of pretreatment B cell numbers. For comparison the level of B cell depletion is shown when a single 250-μg dose of an isotype control version of rituximab (Rit m2a) is used in congenic CD20 Tg mice. (F) WT BALB/c mice received 0.5 mg AT130-2 i.v. daily for 1, 2, or 3 d and the spleens were excised on days 2, 3, or 4, respectively. Splenocytes were stained for B cells (CD19 and B220), macrophages (F480 and CD11b), or DCs (CD11c and MHC class II) and analyzed by flow cytometry. Data are representative of at least three similar experiments, where $n = 4–5$ animals/group. Error is expressed as ± SEM.

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the effects of mAb consumption and provide effective immuno-
therapy, mice were treated with either one, two, or three 0.5-mg
doses of anti-FcγRIIB mAbs. As expected, and in contrast to the
effects in WT mice (Fig. 3A), we were able to demonstrate a clear
and statistically significant improvement in survival with the anti-
FcγRIIB mAbs (Fig. 7A). Likewise, the anti-FcγRIIB mAbs were
also able to further augment immunotherapy of anti-idiotype mAb
in the FcγRIIB2/2 model (Fig. 7B). In some cases, multiple doses
of AT130-2 in combination with anti-idiotype mAbs resulted in
long-term survival (Fig. 7B).

As B cells are a potential sink for the anti-FcγRIIB mAbs, we
subsequently investigated whether deleting B cells might augment
immunotherapy. First, to determine whether the absence of B cells
would improve mAb half-life in vivo, we depleted host B cells
from CD20 Tg mice using Rit m2a and monitored serum mAb
levels. This resulted in enhanced half-life of AT130-2 (Fig. 7C).
Next, anti-FcγRIIB mAb was combined with anti-idiotype mAb
and administered to WT (Fig. 7D, left panel for comparison) or
B cell-depleted mice. Under these circumstances depletion of
B cells lead to improved survival (Fig. 7D, right panel), although
not comparable to that seen in FcγRIIB2/2 mice.

Discussion
mAb immunotherapy is a rapidly evolving treatment modality, with
multiple new mAbs entering the clinical arena each year (57–59).
Surprisingly, given the almost exponential rate of new mAb reagents,
relatively little is still known regarding what makes an effective target
Ag when developing mAbs to recruit cytotoxic effectors. Currently,
CD20 and CD52 stand out as the best available.

In the present study, we have examined the therapeutic potential of
manipulating inhibitory FcγRIIB (CD32B). Initially, we in-
vestigated the effect of deleting FcγRIIB in an mAb immuno-
therapy model of lymphoma and confirmed that lack of FcγRIIB
augmented the ability of anti-idiotype to treat BCL1 lymphoma-
bearing mice and for anti-CD20 mAb to deplete normal B cells,
respectively. Our results are in keeping with the seminal findings

FIGURE 4. Anti-FcγRIIB mAbs are modulated from the cell surface both in vitro and in vivo. (A) WT or γ-chain−/− BALB/c mice were inoculated with 2 × 10⁷ fresh BCL1 cells i.v. on day 0. Mice received 0.5 mg anti-FcγRIIB (AT130-2), anti-idiotype (Mc106A5), or isotype control (WR17) i.p. on day 4. Spleens were excised 48 h later and stained with CD19-allophycocyanin (top right panel) and gated on live cell events (top left panel) to identify B cells. Surface Ag expression and surface-accessible mAbs were then determined by flow cytometry as detailed in Materials and Methods. Maximal receptor expression was determined on control-treated samples. (B) Purified B cells, macrophages, and nBCL1 lymphoma cells were treated with 10 µg/ml anti-FcγRIIB (AT130-2) or anti-idiotype (Mc106A5) and then cultured in vitro for 0–24 h. Cells were washed once and remaining surface Ag was detected as in (A). Surface expression was then calculated as a percentage of expression at 0 h. (C) Purified CD20 Tg B cells were incubated in vitro with Rit m2a-Alexa 488 (Rit-488) and Tosit-Alexa 488 (Tosit-488) mAbs, as well as AT130-2-Alexa 488 (5 µg/ml) for 2–24 h. Cells were washed once and then incubated in the presence or absence of anti-Alexa 488-quenching Ab. The fluorescence remaining after quenching indicates the proportion of internalized mAbs. (D) Malignant (Raji and Daudi) or normal B cells from PBLs were treated with 10 µg/ml AT10, IV.3, KB61 (anti-human FcγRII), or M15/8 (anti-IgM) and then cultured in vitro for 0–24 h. Surface Ag was determined as in (A) and (B). In (A) data are representative of at least three similar experiments, where n = 3 animals/group. In (B)–(D) data are representative of least three independent experiments, where n = 3 samples/group. Error is expressed as ±SEM.
of Clynes et al. (7), albeit we observed less marked enhancement of the therapeutic activity with the anti-idiotype mAb. Those authors showed that mAb therapy of B16 melanoma by TA99 and BT474MI human breast cancer cells by 4D5 and trastuzumab in FcγRIIB2/2 mice abrogated tumor growth far more efficiently than did WT littermate controls. Why the BCL1 model should be less readily augmented is not known but likely reflects the different models used. For example, growth in the B16 model was examined by scoring metastases after day 14, whereas in this study we established the effect of the mAb on overall animal survival in WT and FcγRIIB2/2 mice. Furthermore, in the B16 model, mAb treatment occurred on the same day as administration of the tumor load, which might have prevented the cells from engrafting in the lungs, where in contrast in the present study we treated mice at least 4 d after tumor inoculation. Interestingly, others have also demonstrated little improvement in mAb therapy in FcγRIIB2/2 mice. Takeda et al. (60) treated TRAIL-sensitive 4T1 murine mammary carcinoma with an anti-mouse anti-DR5 mAb (MD5-1). They showed that MD5-1 almost completely abrogated tumor growth in WT mice, but that this therapy was not improved in FcγRIIB−/− mice, perhaps because it relies on pro-apoptotic signaling rather than on cytotoxic effectors. Very recent findings indicate that this apparent discrepancy may relate to the requirement of certain classes of mAb to be cross-linked on the cell surface for FcγRIIB (61–63). The second model Clynes et al. used was a xenograft model of human breast cancer where cells were injected s.c. into nude mice and treated with 4D5 or trastuzumab. Clearly, nude and SCID mice lack components of the adaptive immune system, and so do not mimic the complex relationship between the tumor and its microenvironment. Furthermore, the human breast cancer cells may stimulate a nonphysiological anti-human response within the mouse through the remaining innate immune effectors, which may alter the extent of FcγRIIB involvement, potentially providing more impressive responses.

We subsequently explored the use of mAbs directed toward FcγRIIB to elicit and augment immunotherapy. These mAbs were specific solely for FcγRIIB, elicited a number of potentially important effector functions such as PCD (32) and phagocytosis, and yet were unexpectedly poor in deleting target cells in a number of immunotherapy models, including mouse models of lymphoma, myeloma, and normal B cell depletion. This is in stark contrast to the levels of efficacy reported by Rankin et al. (29) with an anti-human FcγRIIB mAb. In those studies, the human Burkitt’s lymphoma cell line Daudi was injected into immunodeficient nude mice with the mAb given either on day 0 or after 7 d. In both cases, impressive tumor retardation was demonstrated, with the anti-FcγRIIB mAb (h2B6) inducing greater antitumor effects than the current gold standard rituximab. In addition to the caveats inherent in treating tissue-adapted human cell lines in immunodeficient mice, there are a number of additional issues relating to this approach. First, unlike CD20, FcγRIIB is not B cell–specific and is expressed on a number of other lymphoid cell types such as
plasma cells, macrophages, DCs, Kupffer cells, Langerhans cells, basophils, mast cells (4), sinusoidal lining cells in the liver (64), dermal microvascular endothelial cells (65), and placent al villus endothelium (66). Furthermore, in the xenograft model, FcYRs are not blocked by endogenous IgG and the endogenous host cells do not express the human target, meaning that all of the available mAb injected is available for binding to the few tumor cell targets injected.

In relation to our phagocytosis experiments, it was interesting that blocking macrophage FcYRIIB augmented activity. It is known that coaggregation of FcYRIIB with activatory FcYRs results in the phosphorylation of the FcYRIIB ITIM, leading to activation of SHP-1 and SHIP-1 (67, 68), dephosphorylation of Syk, PI3K, and Rac, and inhibition of cell activation and phagocytosis (69-71). By preventing coligation of the activatory FcYRs with FcYRIIB it is likely that anti-FcYRIIB mAbs prevent the appropriate recruitment of these phosphatases to the intracellular domains of the activatory FcYRs, thus allowing uninhibited phagocytosis to occur. This is despite the fact that the blocking mAb used (AT130-5) has previously been shown to phosphorylate FcYRIIB, trigger activation of SHIP-1 and SHP-1, and inhibit BCR signaling responses (32). These data presumably indicate that preventing coaggregation of FcYRIIB with activatory FcYR in macrophages is the key factor in preventing inhibition of phagocytosis through FcYRIIB. They may also indicate that the inhibitory signaling induced by the anti-FcYRIIB mAb is only transient and not sufficiently potent to block phagocytosis.

Our subsequent experiments revealed that FcYRIIB expression on the host cells is a major limitation toward immunotherapy with anti-FcYRIIB mAb, at least in the mouse. As such, the anti-FcYRIIB mAbs were able to elicit significant target cell depletion and immunotherapy of lymphoma in FcYRIIB-/- but not FcYRIIB-replete hosts. At least in part, the reduction in immunotherapy was due to the loss of the mAb from the serum, presumably through mAb consumption in WT mice, similar to that reported by us previously for type I anti-CD20 mAb (47).

Our studies have revealed a role for B cells in anti-FcYRIIB mAb consumption. However, serum mAb was not entirely rescued in the FcYRIIB-BALB/c mice on day 0. Mice reconstituted with FcYRIIB-/- BALB/c mice on day 0. Mice reconstituted with or without 10^7 FcYRIIB-expressing targets in FcYRIIB-/- mice (Fig. 7B), providing further evidence that other cell types are consuming mAbs in vivo. Intriguingly, deletion of B cells with anti-CD20 mAbs provided a more substantial improvement in serum half-life of anti-FcYRIIB mAb than seen in FcYRIIB-/-CD19Cre mice. Potential ly, this difference is attributed to the engagement of FcYRIIB on other endogenous cell types by the anti-CD20 mAb-opsonized B cells during the deletion process. In this setting, deletion of B cells was able to augment the efficacy of combining anti-FcYRIIB and anti-idiotypic mAbs in the BCL-2 model (Fig. 7D). However, the improvement in survival seen following combination therapy was not as pronounced as that seen when anti-idiotypic mAb was administered in the FcYRIIB-/- mice (Fig. 7B), providing further evidence that other cell types are consuming mAbs in vivo.

As with type I anti-CD20 mAb, FcYRIIB mAb consumption appears linked to receptor modulation. The current data confirm that AT130-2 and AT130-5 reduce cell surface levels of FcYRIIB and that this reduction occurs because the receptor is internalized. On which cells and through which receptor (FcYRIIB-1 or -2) the modulation is occurring is the subject of our ongoing studies. Previously, it has been reported that the FcYRIIB-1 isoform, expressed predominantly on B cells, does not internalize, whereas the FcYRIIB-2 isoform expressed on macrophages and DCs does (51, 52). However, in our in vitro and in vivo experiments we noted clear modulation of the surface-bound mAb and receptor in both normal and malignant B cells as well as macrophages and confirmed the expected expression pattern of FcYRIIB isoforms in these cells. Note that the previous conclusions were based on experiments examining effects of IC binding (53, 73) as opposed to direct receptor engagement by mAbs as in this study, where both Fab and Fc engagement are possible. In this scenario, as we previously proposed (32), higher levels of receptor cross-linking may occur, resulting in endocytosis. Furthermore, those initial experiments were performed using human FcYRIIB constructs expressed in an FcYRIIB-deficient derivative of the mouse lymphoma cell line A20 (IAIL6), or in Chinese hamster ovary and COS-1 transfectants containing mouse FcYRIIB. Additionally, the so-called nonendocytic FcYRIIB-1 isoform was previously studied during a very short time scale (within 30 min) (53), whereas in this

**FIGURE 6.** Anti-FcYRIIB mAbs deplete FcYRIIB-expressing targets in FcYRIIB-/- mice. (A) WT (target [T]) and FcYRIIB-/- nontarget (NT) splenocytes were labeled with 5 and 0.5 μM CFSE, respectively, and injected i.v. into WT and FcYRIIB-/- BALB/c mice on day 0. Mice received 100 μg or 0.5 mg AT130-2, AT128 m2a, or irrelevant control (Irr; WR17) i.v. on day 1. Twenty-four hours later, spleens were excised and stained with anti–CD19-allophycocyanin before analysis by flow cytometry. The ratio of CD19+ target/nontarget cells was determined and expressed as a normalized target/nontarget cell ratio. Data represent three experiments, where n = 3 mice/group. ***p < 0.001. (B) Splenocytes from (A) were incubated with or without 10 μg/ml unconjugated anti-FcYRIIB prior to incubation with anti-mouse Fc-PE as previously described. Maximal receptor expression was determined on control-treated samples. Target cells were identified as in (A). Data are representative of three similar independent experiments.
study, we examined receptor modulation at 2, 6, and 24 h, perhaps in part explaining the difference in these studies. Although Clynes and colleagues (74) showed that FcγRIIB on mouse DCs is competent to internalize IC, uniquely on plasmacytoid DCs, it is still unclear from the existing literature whether murine FcγRIIB on murine cells possesses the same endocytic properties as described for the human receptor. There is a subtle difference between the structures of murine and human FcγRIIB, demonstrated by a shortened cytoplasmic tail of the human receptor (51), possibly accounting for the proposed differences in endocytosis. Alternatively, the observed differences in internalization may relate to the species that is, mouse versus human B cells. In support of this notion, we have observed that hCD20 Tg mouse B cells appear to internalize anti-CD20 mAb more rapidly than their normal human B cell counterparts (47). Furthermore, our recent unpublished work using the A20 IAII.6 cells has indicated that these mouse cells also internalize surface mAb unusually rapidly (V. Sha, A. Vaughan, and A. Roghanian, unpublished data).

It was previously reported that levels of FcγRIIB expression do not alter in human lymphoma cell lines after treatment in vivo with a humanized anti-FcγRIIB mAb. Therefore, the modulation we detect on the normal and malignant lymphoma cells after treatment with murine anti-FcγRIIB mAbs both in vitro and in vivo may indeed be a species-dependent observation. Potentially, the lack of modulation reported by Rankin et al. (29) may reflect the cell line nature of the human tumor cell assessed, as we also observed that cell lines routinely modulated type I anti-CD20 mAbs relatively slowly, compared with primary tumor cells (47). However, in our own studies, using anti-human FcγRIIB mAb on both cell lines and primary B cells, we also observed that all three of the mAbs tested did not internalize, in agreement with the data reported by Rankin et al. (29) and in contrast to our results with anti-mouse FcγRIIB mAbs. As discussed above, whether these data reflect inherent differences in the human and mouse receptors, differences in the mouse and human cells or differences in the individual mAbs remain to be determined and is the subject of
our ongoing studies. In an attempt to address this question, we are currently generating transgenic mice that express either the human FcγRIIb alone, or in the presence of the mouse receptor. Using these mice we will be able to directly address whether the human FcγRIIb modulates when bound by mAb (in vitro and in vivo) in primary mouse cells and observe whether it results in rapid consumption of the anti-human FcγRIIb mAbs in vivo. Clearly, the findings from these experiments will have important consequences for the use of these reagents in the clinic.

In summary, in this study we have assessed the ability of FcγRIIb-specific mAbs to treat fully syngeneic mouse tumor models and concluded they are ineffective due to their rapid consumption by FcγRIIb-expressing cells of the host alongside target cell internalization. This novel and unexpected mode of resistance highlights the need to carefully examine the mechanisms of action and resistance with therapeutic mAbs. Furthermore, these data are in marked contrast to those reported with mAbs directed to human FcγRIIB in xenograft studies, and so it remains to be seen whether similar limitations will exist in human cells. The current in vitro data indicate a species-specific difference in the activity of FcγRIIB in mice and humans that provides optimism that anti-human FcγRIIB may not be affected by the same issues, although this requires examination in appropriate model systems and the molecular details remain to be elucidated.

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Disclosures
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