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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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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 Fcγ cells. Unfortunately, suitable mAb reagents capable of specifically recognizing 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, giving the potential to become modulated from the cell surface.

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/III 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-week-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 5T3 mysymetoma (gift from Dr. N. Savelyeva) were maintained in vivo 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 PPL 30/2450 and 30/2451. PI 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-MA [50 µM], glutamine [2 mM], pyruvate [1 mM], penicillin and streptomycin [100 IU/ml], fungizone [2 mg/ml], and 20% FCS [Myoclonal]) (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 (Myoclon) (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 (BMDMs) 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 cell-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. Fab(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 AT130-5 are of mouse origin, whereas AT130-5 and AT128 are of IgG1 isotype. The anti-human FcγRIIB mAbs AT10 (41), IV3 (42), and KB61 (43) were previously described. Rituximab was gifted by Southamton General Hospital oncology pharmacy. Rit m2a was produced as described previously (33). The anti-mouse CD20 mAb 18B2 m12a was made by combinatorial and expressed in the two polypeptide chains in Chinese hamster ovary cells (44). Anti-idiotypic 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 FACSDiva (both BD Biosciences). To assay anti-FcγRIIB mAb (mouse IgG2a subclass) concentrations, sera were incubated with πBCL1, lymphoma cells (FcγRIIB+) and cell-bound anti-FcγRIIB was detected using PE-labeled goat anti-mouse Fc (Jackson Immunoresearch Laboratories) with a standard curve derived 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) Fab(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-C14 fusion protein diluted 1:2 with PBS/Tween 20 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 Fcγ-R-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 × 106) were injected into female 8- to 12-week-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-idiotypic) 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 detailed 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 represented as percentage surface-accessible anti-FcRIIB, which is inversely proportional to the amount of mAb internalized.

RT-PCR for FcγRIIB isoforms

Purified B cells or BMDMs were generated as 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 TCC AGC AGG TCT TCA CCA GTA TTG-3', reverse, 5'-TGC TTG AGA AGT GAG TAG GTG ATC GTA TTC-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 × 10^5 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-F4/80 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 Ab (AbD Serotec) was used 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 × 10^7), 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 (2–4 mice/group) received 0.5 mg of either an irrelevant isotype control (WR17) or anti-Fcγ mAb (AT130-2, AT130-5 also resulted in augmentation of phagocytosis compared with WT mice. BCL1 cells (1 × 10^7) were injected i.v. into C57BL/6 KA mice on day 0. Mice were then culled on day 1 and splenocytes were removed and assessed by flow cytometry for surface expression of the receptor along with surface-accessible mAbs as described above.

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 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 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 (ablatted 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).

FIGURE 1. Anti-idiotype BCL1 immunotherapy shows improved efficacy in FcγRIIB−/− compared with WT mice. BCL1 cells (1 × 10^7) were inoculated i.v. into WT or FcγRIIB−/− BALB/c mice (day 0). Mice then received 125 μg/day control (Mc39/12) or anti-idiotype (Mc106A5; anti-id) mAbs on days 4–7 and survival was monitored. Data represent a typical result from three independent experiments where n = 4–5 mice/group. Median time of survival was increased following anti-idiotype treatment in FcγRIIB−/− compared with WT mice (p < 0.05).
therapy. Given our previous results demonstrating potent antitumor activities in vitro (32), we investigated the potential of our agonistic (AT130-2) and antagonistic (AT128 m2a) Abs (both m2a isotype, which would favor immunotherapy) in vivo. Somewhat surprisingly, the anti-FcγRIIB Abs alone did not result in any substantial improvement in survival in lymphoma (BCL1 or A31) or myeloma (5T-33) models (Fig. 3A–C), although FcγRIIB was well expressed 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-FcγRIIB Abs did not augment the anti-idiotypic immunotherapy in the BCL1 model (Fig. 3D), previously shown by us to be enhanced in the FcγRIIB−/− mice (Fig. 1). We speculated that the tumor models used might have some additional regulatory mechanism able to counteract the anti-FcγRIIB Abs and so examined the ability of the anti-FcγRIIB Abs to deplete normal B cells in vivo using the hCD20 Tg mouse model. A 0.5-mg dose of the anti-FcγRIIB Abs was unable to elicit any substantial depletion of peripheral B cells (Fig. 3E), splenic B cells, macrophages, or DCs, even when the treatment was repeated 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-FcγRIIB Abs 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-FcγRIIB Abs. 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.

FcyRIIB mAb is rapidly lost from the serum through modulation

We previously reported that the efficacy of the type I anti-CD20 mAb rituximab (and its mouse-optimized m2a derivative Rit m2a) becomes limited in vivo due to its modulation from the target cell surface and consumption from the sera (47). Additionally, we recently observed that FcγRIIB is a key regulator of this internalization process (48), leading us to hypothesize that FcγRIIB might also be rapidly internalized upon binding. To address this we transferred BCL1 lymphoma cells into recipient mice, allowed tumor to establish in the spleens, then treated with anti-FcγRIIB Abs, and at 48 h measured the level of FcγRIIB and mAbs on the surface of the splenic tumor cells. Both the anti-FcγRIIB Abs and the receptor were rapidly lost from the cell surface (Fig. 4A). Importantly, the same response was observed in γ-chain−/− mice, indicating that this loss was independent of activatory FcyRs and was therefore likely not a consequence of the shaving phenomenon reported by Beum et al. (49), although it has recently been proposed that shaving may also be driven by FcγRIIB (50) (Fig. 4A). Similar modulation was observed in the A31 model (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 determine whether the same phenomenon was occurring we investigated lymphoma cells as well as normal B cells and BMDMs for their ability to internalize FcγRIIB 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). Previously, it has been reported that the FcγRIIB-1 isoform, expressed predominantly on B cells, does not internalize, whereas the FcγRIIB-2 isoform expressed on macrophages does (51–54). 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. Therefore, we assessed the nature of receptors expressed in these cell types by RT-PCR and confirmed the expected pattern of expression with B cells and tumors expressing predominantly the FcγRIIB-1 isoform and macrophages the FcγRIIB-2 isoform (Supplemental Fig. 3).

We also examined the potential internalization of anti-human FcγRIIB Abs on human cells. None of the three anti-human FcγRIIB Abs 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-FcγRIIB Abs (AT130-2) or anti-idiotypic mAbs in tumor-bearing mice from Fig. 4A. These experiments revealed that anti-FcγRIIB Abs 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 FcγRIIB (Supplemental Fig. 2A, 2B) and also becomes internalized upon engagement (55, 56). These data indicate that the engagement and modulation of the less highly expressed FcγRIIB on the tumor is unlikely to explain the precipitous drop in sera mAb levels. Instead, they indicate that FcγRIIB expressed on other cells may play a dominant role, presumably through a similar internalization process. This hypothesis would also explain the inability of the anti-FcγRIIB mAbs to deplete host cells efficiently (Fig. 3F and data not shown). To examine this specifically we treated WT, FcγRIIB−/−, and γ-chain−/− mice (which retain FcγRIIB expression) with AT130-2 and deter-
A31 were treated with 125I to increase survival of tumor-inoculated mice. 18B12 did improve survival of BCL1-inoculated mice. 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 significantly reverse this effect (Fig. 5C). Genetic deletion of RIIB on B cells (FcγRIIBfl/flCD19Cre+ mice) revealed that anti-FcγRIIB mAbs are efficacious in FcγRIIB competent mice, whereas mAb levels remained far higher in the absence of the receptor (FcγRIIB−/− mice) (Fig. 5B). This is in contrast to the hCD20 Tg model, where endogenous expression of the hCD20 throughout the B cell compartment does not affect the ability of the mAb to deplete target cells or reduce its half-life to such an extent (Ref. 47 and data not shown). Increasing the dose of anti-FcγRIIB mAbs from a single 250-μg dose to multiple doses on sequential days was unable to significantly reverse this effect (Fig. 5C). Genetic deletion of FcγRIIB on B cells (FcγRIIB−/−CD19Cre+ mice) revealed that B cells only make a modest contribution to mAb consumption in vivo with a small effect on day 1 (Fig. 5D).

Anti-FcγRIIB mAbs are efficacious in FcγRIIB−/− mice

Having established that the anti-FcγRIIB mAbs were being consumed by endogenous cells, we next assessed whether FcγRIIB-expressing target cells could be depleted in the absence of FcγRIIB in the host. First, using an in vivo depletion assay (47) we transferred CFSE-labeled FcγRIIB+ splenocytes and nontarget FcγRIIB−/− splenocytes into either WT or FcγRIIB−/− mice and examined the ability of the target cells to become depleted by the anti-FcγRIIB mAbs. These experiments revealed that anti-FcγRIIB mAbs were only able to efficiently deplete FcγRIIB+ target B cells in the FcγRIIB−/− recipients (Fig. 6A). Moreover, increasing the dose of mAb from 100 to 500 μg did not yield appreciable depletion in the WT host. Furthermore, by assessing the remaining CFSE-labeled target cells 24 h after treatment, we were able to demonstrate that the modulation of FcγRIIB was much less in WT compared with FcγRIIB−/− recipient mice. This indicates that in the context of an FcγRIIB competent mouse, the anti-FcγRIIB mAb is being consumed before it reaches the target B cells (Fig. 6B). Interestingly, there was little discrimination between the agonistic or antagonistic nature of the anti-FcγRIIB mAbs in these assays, with both AT130-2 and AT128 m2a giving equivalent in vivo depletion of targets.

Subsequently, having demonstrated that mAb consumption by the cells of the host mouse could be the basis for impaired depletion of target cells, we repeated our immunotherapy experiments with BCL1 tumor cells in FcγRIIB−/− mice. In an attempt to overcome
the effects of mAb consumption and provide effective immunotherapy, 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 FcγRIIB−/− 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 FcγRIIB−/− 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 FcγRI (CD32B). Initially, we investigated the effect of deleting FcγRIIB in an mAb immunotherapy model of lymphoma and confirmed that lack of FcγRIIIA 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...
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 FcγRIIB 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 FcγRIIB 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 FcγRIIB 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 by 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 mAbs. 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...
injected i.v. into WT and FcRRIIB splenocytes were labeled with 5 and 0.5 μM CFSE, respectively, and injected i.v. into WT and FcRRIIB−/− BALB/c mice on day 0. Mice received 100 μg or 0.5 mg AT130-2, AT128 mAb, or irrelevant control (Irr; WR17) i.v. on day 1. Twenty-four hours later, spleens were excised and experiments, where

**FIGURE 6.** Anti-FcRRIIB mAbs deplete FcRRIIB-expressing targets in FcRRIIB−/− mice. (A) WT (target [T]) and FcRRIIB−/− nontarget (NT) splenocytes were labeled with 5 and 0.5 μM CFSE, respectively, and injected i.v. into WT and FcRRIIB−/− 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-FcRRIIB 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.

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 placental villus endothelium (66). Furthermore, in the xenograft model, FcRRIIB is 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 FcRRIIB augmented activity. It is known that coaggregation of FcRRIIB with activatory FcγRs results in the phosphorylation of the FcRRIIB 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 FcγRs with FcRRIIB it is likely that anti-FcRRIIB mAbs prevent the appropriate recruitment of these phosphatases to the intracellular domains of the activatory FcγRs, thus allowing uninhibited phagocytosis to occur. This is despite the fact that the blocking mAb used (AT130-5) has previously been shown to phosphorylate FcRRIIB, trigger activation of SHIP-1 and SHIP-1, and inhibit BCR signaling responses (32). These data presumably indicate that preventing coaggregation of FcRRIIB with activatory FcγR in macrophages is the key factor in preventing inhibition of phagocytosis through FcRRIIB. They may also indicate that the inhibitory signaling induced by the anti-FcRRIIB mAb is only transient and not sufficiently potent to block phagocytosis.

Our subsequent experiments revealed that FcRRIIB expression on the host cells is a major limitation toward immunotherapy with anti-FcRRIIB mAb, at least in the mouse. As such, the anti-FcRRIIB mAbs were able to elicit significant target cell depletion and immunotheraphy of lymphoma in FcRRIIB−/− but not FcRRIIB-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 1 anti-CD20 mAb (47).

Our studies have revealed a role for B cells in anti-FcRRIIB mAb consumption. However, serum mAb was not entirely rescued in the FcRRIIB−/−CD19Cre+ mice, indicating that other FcRRIIB+ cell types (such as macrophages, DCs, Kupffer cells, and certain endothelial cells) (72) may be additional consumers of mAb in vivo. Intriguingly, deletion of B cells with anti-CD20 mAbs provided a more substantial improvement in serum half-life of anti-FcRRIIB mAb than seen in FcRRIIB−/−CD19Cre+ mice. Potentially, this difference is attributed to the engagement of FcRRIIB 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-FcRRIIB and anti-idiotype mAbs in the BCL1 model (Fig. 7D). However, the improvement in survival seen following combination therapy was not as pronounced as that seen when anti-idiotype mAb was administered in the FcRRIIB−/− mice (Fig. 7B), providing further evidence that other cell types are consuming mAbs in vivo.

As with type 1 anti-CD20 mAb, FcRRIIB mAb consumption appears linked to receptor modulation. The current data confirm that AT130-2 and AT130-5 reduce cell surface levels of FcRRIIB and that this reduction occurs because the receptor is internalized. On which cells and through which receptor (FcRRIIB) engagement is attributed to the engagement of FcRRIIB on other endogenous B cells, does not internalize, whereas the FcRRIIB-2 isoform expressed on macrophages and DCs (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 FcRRIIB 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 FcRRIIB constructs expressed in an FcRRIIB-deficient derivative of the mouse lymphoma cell line A20 (IAIL.6), or in Chinese hamster ovary and COS-1 transfectants containing mouse FcRRIIB. Additionally, the so-called nonendocytic FcRRIIB-1 isoform was previously studied during a very short time scale (within 30 min) (53), whereas in this
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 expressed 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 IAIL.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 FcγRII 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 research.
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 controls 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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