FcγRIIB controls antibody-mediated target cell depletion by ITIM-independent mechanisms

Alexander P. Simpson[1*], Ali Roghanian[1,4*], Robert Oldham[1], H.T. Claude Chan[1], Christine A. Penfold[1], Hyung J. Kim[1], Tatyana Inzhelevskaya[1], C. Ian Mockridge[1], Kerry L. Cox[1], Yury D. Bogdanov[1], Sonya James[1], Alison L. Tutt[1], Daniel Rycroft[2], Peter Morley[2], Lekh N. Dahal[1,‡], Ingrid Teige[3], Björn Frendeus[3,8], Stephen A. Beers[1,8] and Mark S. Cragg[1,4,8]

[1]Antibody and Vaccine Group, Centre for Cancer Immunology, School of Cancer Sciences, University of Southampton Faculty of Medicine, Southampton, Hampshire, SO16 6YD, UK.

[2]Biopharm Discovery, GSK, Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY

[3]BioInvent International AB, Sölvegatan 41, 22370 Lund, Sweden

[4]Institute for Life Sciences, University of Southampton, Southampton, Hampshire, SO17 1BJ, UK;

[*] These authors contributed equally to the manuscript

[‡] Current address: Molecular and Clinical Pharmacology, Institute of Translational Medicine, Liverpool University, Liverpool, UK

[8]Corresponding authors: Bjorn.Frendeus@bioinvent.com, sab@soton.ac.uk and msc@soton.ac.uk

Running title: FcγRIIB ITIM signaling is dispensable for preventing antibody-mediated depletion

Summary

Many therapeutic antibodies deplete target cells and elicit immunotherapy by engaging activating Fc gamma receptors (FcγR) on host effector cells. These antibodies are negatively regulated by the inhibitory FcγRIIB (CD32B). Dogma suggests inhibition is mediated through the FcγRIIB immunoreceptor tyrosine-based inhibition motif (ITIM), negatively regulating immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling from activating FcγR. To assess this, we generated experimental models expressing human (h)FcγRIIB on targets or effectors, lacking, or retaining ITIM signaling capacity. We demonstrate that signaling through the hFcγRIIB ITIM is

dispensable for impairing monoclonal antibody (mAb)-mediated depletion of normal and malignant murine target cells through three therapeutically relevant surface receptors (CD20, CD25 and OX40) impacting immunotherapy. We demonstrate that hFcγRIIB competition with activating FcγRs for antibody Fc, rather than ITIM signaling, is sufficient to impair activating FcγR engagement, inhibiting effector function and immunotherapy.

Significance

Direct targeting (cytotoxic) monoclonal antibodies (mAb) provide treatments for many cancers. FcγRIIB is the sole inhibitory IgG receptor and known to reduce their efficacy. How this inhibition is mediated and may be best overcome is unclear. We demonstrate that FcγRIIB mediates its effects through competition with other FcγRs and that Fc-null FcγRIIB-blocking mAb are optimal when target cells lack FcγRIIB. This knowledge will help guide the development of the next generation of anti-FcγRIIB reagents for optimal mAb immunotherapy in the clinic.

KEYWORDS

Immunotherapy, depletion, FcγRIIB, ITIM, B cells, Treg, monoclonal antibody, Fc receptors

Highlights

- WT but not Fc-null FcγRIIB antibodies deplete FcγRIIB^{+} targets
- ITIM signaling is dispensable for FcγRIIB-mediated inhibition of target cell depletion and impairment of anti-tumor efficacy
- FcγRIIB inhibits mAb-mediated depletion by outcompeting activating FcγR on myeloid effector cells

Introduction

Monoclonal antibodies (mAb) such as rituximab, cetuximab and trastuzumab are an important class of therapeutics, binding directly to tumor cells and evoking their destruction (Scott et al., 2012) (Chan and Carter, 2010). Their mechanism of action is governed by their interactions with Fc gamma receptors (FcγR) (Glennie et al., 2007), with the response modulated by the specific receptor(s) engaged, the Fc valency of the immune complex, the cell types involved and the architecture of the cellular microenvironment (Koenderman, 2019) (DiLillo and Ravetch, 2015).

FcγRs can be grouped into activating or inhibitory variants depending on function (Bruhns, 2012). Activating FcγRs evoke ITAM-mediated signaling to elicit functions such as phagocytosis and cytokine release in response to antibody-coated cells or immune complexes (Getahun and Cambier, 2015). Several elegant studies have highlighted the significance of activating FcγRs in mediating the activity of therapeutic mAb with loss of receptor expression (Clynes et al., 2000) or signaling (de Haij et al., 2010) abrogating mAb-mediated cell depletion. In mice at least, the key FcγR-expressing effector cells derive from the mononuclear phagocyte system, mediating mAb-mediated target cell depletion for a range of targets including CD20, EGFR, gp75, CD25 and OX40 (Arce Vargas et al., 2017; Beers et al., 2010; Bulliard et al., 2014; Grandjean et al., 2016; Gul et al., 2014; Roghanian et al., 2019; Setiady et al., 2010).

The counterpoint to these activating FcγR is the sole inhibitory FcγR, FcγRIIB (FcγRII in mice) (Roghanian et al., 2018). This receptor contains an intracellular immunoreceptor tyrosine-based inhibition motif (ITIM) motif which, upon phosphorylation, acts to reverse the activating pathways initiated by immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors such as activating FcγR and the B cell antigen receptor (BCR) (Getahun and Cambier, 2015). The phosphorylated ITIM provides a docking site for the phosphatases Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1) and SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1). These phosphatases then act to convert PIP_3 to PIP_2 and de-phosphorylate ITAM and ITAM-associated kinases such as Src and Syk (Huang et al., 2003) to attenuate activating FcγR signaling. FcγRIIB is also an important mediator of humoral immunity through its regulation of ITAM signals downstream of the BCR, attenuating B cell activation, proliferation, survival, and differentiation as well as directly impacting affinity maturation and plasma cell survival (Barrington et al., 2002; Ono et al., 1997; Xiang et al., 2007).

Several studies have shown that FcγRIIB can negatively impact the therapeutic efficacy of mAb. For example, genetic deletion of mouse (m) FcγRII was shown to enhance phagocytic function *in vitro* as well as augment mAb-mediated tumor control *in vivo* (Clynes et al., 1999; Clynes *et al.*, 2000). Pre-

clinical and explorative retrospective clinical studies also indicate that human (h)FcγRIIB has additional means of ameliorating direct targeting mAb activity. For example, B cell malignancies that demonstrate a high expression of hFcγRIIB have been correlated with resistance to target cell depletion mediated by type-I anti-CD20 mAb, such as rituximab. Here, hFcγRIIB augments internalization of the CD20:mAb complex through cis-binding on the surface of the B cell (Lim et al., 2011), resulting in increased mAb consumption (Lim *et al.*, 2011), the attenuation of Fc-mediated effector functions (Beers et al., 2008; Tipton et al., 2015b) and reduced mAb persistence (Tipton *et al.*, 2015b). Accordingly, high tumor cell hFcγRIIB expression is linked to poor response to rituximab in follicular lymphoma (Lee et al., 2015), and diffuse large B cell lymphoma (DLBCL) patients (Nowicka et al., 2021).

Taken together, these data support a hypothesis that hFcγRIIB represents an attractive target for overcoming resistance to direct targeting mAb and improving therapeutic responses. We therefore previously generated a panel of mAb capable of targeting hFcγRIIB and showed that they enhanced anti-CD20 mAb-mediated depletion of both normal and malignant B cells *in vivo*, as well as anti-CD52 mAb-mediated depletion of chronic lymphocytic leukemia (CLL) cells (Roghanian et al., 2015).

Although the importance of ITIM signaling has been established for the hFcγRIIB mediated modulation of BCR signaling (Muta et al., 1994; Ono *et al.*, 1997) and the inhibition of ITAM related functions *in vitro* (Daeron et al., 1995), it has no role in the aforementioned hFcγRIIB-mediated mAb internalization process, which was demonstrated to be independent of the hFcγRIIB intracellular tail (Vaughan et al., 2015). Similarly, the ability of hFcγRIIB to cluster and activate immunomodulatory mAb has been demonstrated to be independent of hFcγRIIB signaling (Li and Ravetch, 2013; White et al., 2011). Crucially, the contribution of ITIM-mediated signaling to the inhibition of mAb-mediated cell depletion *in vivo* remains to be determined. To address this and better understand the most effective means of blocking FcγRIIB for enhancing direct targeting mAb efficacy, we generated a range of unique mouse models expressing human and/or mouse FcγRIIB on target or effector cells, lacking or retaining signaling capacity, alongside blocking reagents able to bind FcγRIIB alone or alongside other FcγR. We then deployed these tools to address these issues with respect to clinically relevant receptor targets on B cells and regulatory T cells (Treg).

Results

Impact of FcγRIIB mAb on rituximab-mediated B cell depletion depends on Fc functionality and FcγRIIB expression profile

We previously developed a highly specific fully human IgG1 mAb directed to hFcγRIIB (6G11, 6G, BI-1206), that does not bind to mFcγRII, and showed its ability to augment rituximab-mediated depletion of normal and malignant B cells *in vivo* (Roghanian *et al.*, 2015). We postulated that activity was based upon its ability to engage 3 separate mechanisms: (i) direct Fc:FcγR-mediated cell depletion (with hFcγRIIB itself operating as a direct targeting antigen), (ii) prevention of rituximab internalization, and (iii) blocking hFcγRIIB-mediated inhibition of myeloid effector cells (Roghanian et al., 2016). To delineate the relative importance of these and which FcγRIIB mAb format was optimal, we developed a panel of hFcγRIIB transgenic (Tg) mice and hFcγRIIB mAb, displaying wild-type (WT) or defective (N297Q mutation; herein referred to as Fc-null) Fc domains. We then assessed their ability to deplete $hCD20^{+}$ target B cells both alone and in combination with rituximab in pre-clinical models where hFcγRIIB was present on target cells, effector cells, or both. Using this approach, we were able to measure depletion of the adoptively transferred B cells or tumors in the presence or absence of different FcγRIIB molecules and investigate depletion with a clinically relevant anti-hCD20 mAb.

Initially, we tested systems where hFcγRIIB was expressed only on the target cell, first adoptively transferring $hCD20^{+/-}$ x $hFcγRIIB^{+/-}$ x $mFcγRII^{-/-}$ murine splenocytes into WT C57BL/6J mice (Figure 1A). WT hFcγRIIB mAb (6G) depleted target cells when administered alone and potentiated the therapeutic activity of rituximab (RTX) (Figure 1B). Fc-null hFcγRIIB mAb (6G-Q) did not deplete target B cells when administered alone (due to the absence of a functional Fc) and did not improve depletion in combination with rituximab (Figure 1B). Next, we used a human Raji B cell lymphoma xenograft model and showed that co-administration of Fc-null 6G-Q mAb inhibited the therapeutic activity of rituximab, abolishing tumor control (Figure 1C). These observations with 6G-Q in both systems were in marked contrast to our previous findings with 6G in combination with rituximab where we demonstrated a clear beneficial effect (Roghanian *et al.*, 2015). Finally, we tested the effects of 6G-Q in combination with rituximab in a second xenograft model with primary human CLL cells. In agreement with the models above, co-administration of an Fc-null hFcγRIIB mAb did not improve the depletion mediated by rituximab compared to rituximab alone (Figure 1D).

Next, we assessed the impact of anti-hFcγRIIB mAb in systems where hFcγRIIB was lacking from the target cells and was expressed only on the effector cells of the host. Target $hCD20^{+/-}$ x $mFcγRII^{-/-}$ murine splenocytes (lacking hFcγRIIB) were adoptively transferred into $hFcγRIIB^{+/-}$ x $mFcγRII^{-/-}$ recipients and treated as before (Figure 1E). 6G and 6G-Q mAb treatment alone had no effect on target B cells, whereas rituximab monotherapy resulted in a ~40% reduction of target cells (Figure 1F). Notably, and

in direct contrast to that observed in Figures 1B-D, Fc-null 6G-Q potentiated the depletion of B cells when combined with rituximab, whereas, WT 6G impaired activity. Together, these observations demonstrate that the Fc-functionality of hFcγRIIB mAb has differential and opposing effects on augmenting B cell depletion when directed to target or effector cells. Given the opposing effects of the two hFcγRIIB mAb formats, we next addressed whether they had the potential to stimulate ITIM signaling in relevant immune effector cells. Bone marrow derived macrophages (BMDMs) were stimulated with either 6G or 6G-Q and the phosphorylation of the FcγRIIB ITIM domain was assessed. Treatment of cells with 6G, but not 6G-Q mAb resulted in the phosphorylation of the hFcγRIIB-ITIM (Figure 1G). These data could explain why WT hFcγRIIB mAb but not Fc-null mAb impair the subsequent depleting capacity of rituximab (by delivering hFcγRIIB-mediated inhibitory signaling into myeloid cells). However, these conclusions assume that the observed ITIM-mediated signaling is central to these inhibitory effects and so we explored this directly by generating hFcγRIIB Tg mice carrying a defective ITIM.

Generation and characterization of hFcγRIIB 'NoTIM' mice, lacking a functional ITIM

ITIM defective mice were generated based upon our previous hFcγRIIB Tg construct (Roghanian *et al.*, 2015) with mutation of tyrosine 273 within the ITIM to phenylalanine (Y273F), which impairs interaction with SHIP-1 (Stopforth et al., 2018). To further reduce the potential for signaling we also mutated a second tyrosine in exon 7 (Y254F) proximal to the ITIM (Figure 2A), confirmed by Sanger sequencing (Figure 2B, Figure S1 A-B). We termed this new model 'NoTIM' in reference to the non-signaling activating FcγR 'NOTAM' mouse produced previously (de Haij *et al.*, 2010) and crossed it with C57BL/6J mFcγRII$^{-/-}$ mice to remove the endogenous mouse inhibitory FcγR. Surface expression of the NoTIM hFcγRIIB was confirmed by flow cytometry and immunofluorescence microscopy on relevant cell populations; *i.e.*, B cells and monocytes but not NK cells (Figure 2C, D). Western blot analysis using an agonistic hFcγRIIB mAb (6G08) (Roghanian *et al.*, 2015) demonstrated that ITIM phosphorylation of hFcγRIIB was lost in NoTIM but not hFcγRIIB Tg BMDMs and B cells (Figure 2E). Further downstream analysis showed phosphorylated SHIP-1 in hFcγRIIB Tg B cells but not B cells from NoTIM mice (Figure S1E). B cells from NoTIM mice were also partially impaired in their ability to internalize soluble immune complex (ICs) compared to B cells expressing WT hFcγRIIB (Figure 2F). This agrees with earlier studies showing mFcγRII regulates IC internalization and that the loss of the mFcγRII tyrosines reduced the speed of internalization (Miettinen et al., 1992). The same agonistic hFcγRIIB mAb was also able to reduce anti-IgM-mediated BCR calcium flux in hFcγRIIB Tg but not NoTIM B cells (Figure 2G). Having established a lack of inhibitory FcγR signaling in these mice, next, we assessed the expression pattern

of hFcγRIIB, alongside the remaining mFcγRs, and found hFcγRIIB expression to be comparable to that in our hFcγRIIB Tg mouse (and in humans, Figure 2C, Figure S1 C-D), without significant change to activating mFcγRs in the mice (Figure S1 F-H). Together these data demonstrate that expression of a non-signaling hFcγRIIB was achieved in NoTIM mice in a physiologically relevant manner akin to that in mice expressing a functional hFcγRIIB, facilitating subsequent comparisons.

hFcγRIIB mediated inhibition of anti-mCD20 mAb is not dependent on inhibitory signaling *in vivo*

To determine if ITIM signaling was important for impairing anti-mCD20-mAb-mediated B cell depletion, mice were treated with increasing, stepwise, doses (2, 10, 50 μg) of an anti-mCD20 antibody (clone 18B12) (Ahuja et al., 2007). Both mIgG1 and mIgG2a isotypes were utilized due to their varied activating to inhibitory (A:I) FcγR binding profiles and ratios (Nimmerjahn and Ravetch, 2005), with depletion of circulating B cells then assessed (Figure 3A-B). When using the mIgG1 isotype, which has a modest A:I binding ratio (engaging mFcγRII and mFcγRIII), mFcγRII$^{-/-}$ mice (expressing only activating mFcγRs) were most susceptible to antibody-mediated B cell depletion (Clynes *et al.*, 2000) (Figure 3C). Notably, NoTIM mice, lacking intrinsic inhibitory signaling, were significantly more resistant to anti-mCD20-mediated B cell depletion than mFcγRII$^{-/-}$ mice, with hFcγRIIB Tg and WT C57BL/6J mice displaying an intermediate response after both 10 μg and 50 μg doses (assessed on day 9 and day 23, respectively; Figure 3D). This difference was most evident after the final 50 μg dose on day 23. The signaling-competent hFcγRIIB Tg mice formed two clusters: responders displaying B cell depletion similar to mFcγRII$^{-/-}$ mice and non-responders showing depletion more similar to NoTIM mice (Figure 3D). As previously observed (Roghanian *et al.*, 2015), our hFcγRIIB Tg mice display a mosaic expression pattern (Figure S1G), with penetrance varying between mice. To assess if transgene expression impacted depletion, the percentage hFcγRIIB positivity of each mouse was quantified using flow cytometry and then correlated against peripheral B cell depletion. There was a strong correlation between transgene expression and percentage of remaining peripheral B cells in these mice, indicating a direct relationship between hFcγRIIB expression and inhibition of anti-mCD20-mediated B cell depletion (Figure S2).

mIgG2a can additionally engage mFcγRI and mFcγRIV, displaying a higher FcγR A:I ratio than mIgG1. Accordingly, B cells were depleted more effectively at lower doses with anti-mCD20 mIgG2a in WT mice than the mIgG1 mAb, with WT and mFcγRII$^{-/-}$ mice displaying similar levels of depletion (Figure 3E-F). Nevertheless, clear inhibition of B cell depletion was seen in the NoTIM mice, with hFcγRIIB Tg mice showing intermediate depletion. The impacts on depletion for anti-mCD20 mIgG1 and mIgG2a were also reflected in the number of B cells observed in secondary lymphoid tissues of the spleen and

lymph nodes (Figure 3G-H), with depletion being significantly reduced in NoTIM mice. These data demonstrate that cell surface expression of hFcγRIIB is tightly linked to inhibition of anti-mCD20 mAb-mediated B cell depletion and that ITIM-mediated signaling is not required for impairing the depletion capabilities of mIgG1 and mIgG2a isotypes. Next, we considered the underpinning mechanism.

hFcγRIIB does not adversely impact anti-mCD20 mAb serum exposure

FcγRIIB has been demonstrated to regulate antibody half-life through the removal of small immune complexes, predominantly via the sinusoidal endothelial cells of the liver (Ganesan et al., 2012) . To ascertain if the lack of depletion in the various mouse strains was due to rapid loss of anti-mCD20 mAb from circulation, serum exposure was assessed. mFcγRII $^{-/-}$, hFcγRIIB Tg and NoTIM mice were intravenously injected with 50 µg mAb (mIgG1) and bled at regular intervals to ascertain serum concentrations for comparison with WT C57BL/6J mice (Figure 4A). Within the same mice, the level of B cell depletion was concurrently assessed. Serum levels of antibody remained largely similar across strains (Figure 4B-C) and did not correlate with depletion at 6 or 48h (Figure 4C-D). The observed mAb half-life ($t_{1/2}$), area under the curve (AUC_{0-t}), volume of distribution (V_z) and clearance (CL) indicated that the serum availability of anti-mCD20 mAb was similar across all groups (Figure 4E). Although serum levels remained highest in mFcγRII$^{-/-}$ mice, the nadir of all animal groups remained above that required for saturation of mCD20 on B cells at 48 hours post-injection (Figure S3A). Together, these data indicate that anti-mCD20 serum exposure does not explain the difference in depletion efficacy between the four mouse models examined. Further supporting this notion, antibody exposure was independent of transgene expression in hFcγRIIB Tg mice (Figure S3B).

hFcγRIIB regulates anti-mCD20 mAb internalization independently of depletion

Antibodies directed to hCD20 can undergo internalization in a manner which is accelerated by co-engagement of hFcγRIIB (Lim *et al.*, 2011), but independent of intracellular signaling (Vaughan *et al.*, 2015). We therefore considered whether rapid internalization of mAb-mCD20 complexes from the B cell surface might explain why NoTIM mice were highly resistant to B cell depletion. An *in vitro* internalization assay using B cells from each mouse model (Figure S3C-D) showed internalization was modest overall, being near absent at 2 hours and ~40% after 24 hours. Although broadly similar levels of internalization were seen, the presence of the mouse or human inhibitory receptor (C57BL/6J, hFcγRIIB or NoTIM) accelerated the internalization of anti-mCD20 mIgG1 to a similar degree, compared to the mFcγRII$^{-/-}$ B cells (Figure S3E). These results are in keeping with previous

observations, showing that hFcγRIIB increased internalization of anti-hCD20 mAb from the B cell surface (Beers *et al.*, 2010; Lim *et al.*, 2011; Vaughan et al., 2014). Consequently, this may explain the small differences in serum exposure seen between mFcγRII$^{-/-}$ and other mouse models but is unlikely to explain the profound differences in B cell depletion observed.

NoTIM effector cells suppress anti-hCD20 mAb-mediated target cell depletion *in vivo*

As described earlier, hFcγRIIB is expressed on both B cell targets and myeloid effector cells. To establish the cell type on which FcγRIIB exerts its inhibitory effects we devised a series of experiments to separately examine its impact on target or effector cells (akin to those in Figure 1). First, to determine the effects of hFcγRIIB on target cells, splenocytes were isolated from hCD20$^{+/-}$ x NoTIM mice (targets) and adoptively transferred alongside mFcγRII$^{-/-}$ splenocytes (non-targets) into mFcγRII$^{-/-}$ recipients (Figure 5A). These mice were dosed with antibodies to block FcγRIIB, with either WT (6G) or null (6G-Q) Fc domains, and then treated with rituximab before measuring the target:non-target (T:NT) ratio. To assay for potential impacts in different locations, the blood, spleen, and bone marrow were examined. Rituximab resulted in a robust depletion of target cells from the blood and spleen, with similar effects in the bone marrow (Figure 5B and S4A, respectively). 6G also resulted in potent depletion of target B cells expressing the NoTIM hFcγRIIB, both alone and in combination with rituximab. In contrast, 6G-Q monotherapy had no impact on B cell depletion and did not improve target depletion in combination with rituximab. These data indicate that 6G enhances target cell depletion through opsonization of the hFcγRIIB^{+} target cell. In contrast, 6G-Q which cannot interact with activating mFcγRs also cannot mediate or promote cell clearance through target cell FcγRIIB (Figure S4A), as also shown in WT hFcγRIIB mice in Figure 1D.

Next, we assessed the impact of the NoTIM hFcγRIIB when expressed only on effector cells. Accordingly, splenocytes were isolated from hCD20$^{+/-}$ x mFcγRII $^{-/-}$ mice (targets) and adoptively transferred alongside mFcγRII$^{-/-}$ splenocytes (non-targets) into mFcγRII$^{-/-}$ or NoTIM mice (Figure 5C). These mice were treated in a similar manner as before (Figure 1B-D and 5B) and the T:NT ratio determined. As expected, rituximab monotherapy was effective in mFcγRII$^{-/-}$ mice (Figure 5D; blue circles). In contrast, rituximab monotherapy in NoTIM mice was less efficient, depleting fewer target B cells, (purple triangles) indicating that inhibition of target cell depletion arises from expression of the NoTIM receptor on effector cells (Figure 5D and S4B). The addition of 6G to rituximab did not improve target cell depletion in NoTIM mice. However, the addition of the Fc-null 6G-Q significantly enhanced rituximab depletion compared to rituximab monotherapy to levels equivalent to those in mFcγRII$^{-/-}$ mice (Figure 5C/D and S4B). These data replicate those shown for the WT hFcγRIIB receptor

(Figure 1F) and demonstrate that overcoming hFcγRIIB-mediated inhibition of depletion on effector cells is only efficiently achieved using an Fc-null hFcγRIIB-blocking mAb.

Previously, we hypothesized that the inability of WT FcγRIIB blocking mAb to enhance target cell depletion may be due to its Fc-mediated inhibitory signaling in myeloid effector cells (Figure 1G); however, in the NoTIM mouse this is not possible due to the mutated ITIM. In the absence of a possible signaling effect and in light of the observation that inhibition correlates with surface expression, we considered whether hFcγRIIB impaired depletion by binding and sequestering the Fc of the opsonizing mAb - preventing engagement of activating receptors. To examine this, the availability of activating mFcγRs on myeloid effector cells was analyzed *ex vivo* after mAb treatment. Treatment of NoTIM mice with an isotype control antibody or rituximab did not alter mFcγR detection. In contrast, blockade with 6G (monotherapy or in combination with rituximab) greatly reduced the detection of mFcγRIV on splenic macrophages, with small decreases also seen in mFcγRI and mFcγRIII (Figure 5E-F). The effect was not observed with 6G-Q. Similar reductions in activating FcγR detection following 6G, but not 6G-Q treatment was also observed on other potential effector cells ($Ly6G^+$ $Ly6C^+$ neutrophils and $Ly6C^{hi}$ monocytes; Figure S4C-D). We then performed similar experiments in hFcγRIIB Tg mice, showing the same effect (Fig 5G). These data indicate the WT Fc region of 6G occupies the binding site of activating mFcγRs on effector cells, reducing the ability of the rituximab Fc to engage these same activating mFcγRs, decreasing target cell depletion. In contrast, the Fc-null 6G-Q does not block activating mFcγRs, leaving them available to interact with the Fc of rituximab and elicit strong depletion (Figure S4B). Together, these experiments demonstrate that the cell surface expression of hFcγRIIB on myeloid effector cells is responsible for the suppression of anti-CD20 mAb-mediated target cell depletion through a signaling independent mechanism, involving competition for Fc, and preventing engagement of activating mFcγRs.

Targeting of receptors concurrently on target cells and myeloid effectors impairs mAb-mediated depletion *in vivo*

We next considered whether this effect was related solely to targeting FcγRs or to the Fc-mediated blockade of activating FcγRs. To explore this, we performed experiments targeting hCD40 – again when the antigen was on the target alone (B cells) (Figure 6A) or on both target and effector cells (Figure 6B). To achieve this, we transferred splenocytes from hCD40 Tg mice (targets) alongside WT splenocytes (non-targets) into WT C57BL/6J or hCD40 Tg mice, treating them with effective depleting isotypes (mIgG2a and hIgG1) of anti-hCD40 mAb. Depletion of target B cells was robust in WT mice (where the myeloid effector cells lack hCD40) but inefficient in hCD40 Tg mouse recipients (Figure 6C).

Importantly, this was not due to the larger antigenic sink in the hCD40 Tg mice, as the serum levels of available mAb was indistinguishable in both contexts (Figure 6D). To explore the underpinning mechanism, we again examined the availability of activating mFcγRs on myeloid effector cells. As before, we observed that in instances where depletion is blunted (*i.e.*, hCD40 Tg recipients) the staining of mFcγRs, including FcγRIV, was reduced (Figure 6E) but that this was not the case in WT C57BL/6J recipient mice (Figure 6F).

hFcγRIIB impairs depletion of regulatory T cells in a signaling-independent manner

Having made these observations with B cell targets, we next sought to evaluate other clinically relevant cellular targets, namely Tregs. Tregs regulate multiple facets of the immune response and their depletion is an important goal in several immunotherapy strategies (Elpek et al., 2007; Golgher et al., 2002; Onizuka et al., 1999; Rech et al., 2012) with CD25 being a clinically-relevant Treg target (Arce Vargas *et al.*, 2017; Rech *et al.*, 2012; Solomon et al., 2020). Accordingly, mFcγRII$^{-/-}$, hFcγRIIB Tg and NoTIM mice were treated with anti-mCD25 mAb (clone PC61) or an isotype control and then bled at regular time points to examine the kinetics of CD4^{+}FOXP3^{+} Treg depletion (Figure 7A). In agreement with the anti-mCD20 experiments, NoTIM mice were consistently more resistant to Treg depletion than both hFcγRIIB Tg and mFcγRII$^{-/-}$ mice, both from the blood at day 2 and 4 and the spleen at harvest (Figure 7B). Together these data demonstrate an additional target and cell type where mAb-mediated depletion is impaired by hFcγRIIB in an ITIM signaling-independent manner.

hFcγRIIB impairs depletion of cells in a signaling-independent manner with impact on cancer immunotherapy

Next, we considered whether the impaired depletion of these various target cells in the NoTIM mouse would also occur in the tumor microenvironment (TME). To address this, we considered a fourth cell surface target receptor, OX40. It is known that targeting mOX40 can deplete intratumoral Tregs via activating mFcγRs, leading to anti-tumor efficacy (Bulliard *et al.*, 2014) and we recently made similar observations for hOX40 in a knock-in mouse model (Griffiths et al., 2020). We therefore assessed the ability of the anti-mOX40 mAb OX86 mIgG2a to deplete Tregs in the EG7 thymoma model (Figure 7C). As with anti-mCD25, anti-mOX40 mAb depleted Treg efficiently from the blood (Figure 7D) and spleen (Figure 7E) of FcγRII$^{-/-}$ mice. hFcγRIIB Tg mice displayed intermediate depletion levels and NoTIM mice were most resistant to depletion, which was also reflected in the CD8:Treg ratios (Figure 7D-E lower panels). We next assessed Treg depletion from the tumor, with the findings replicating those found in

the blood (Figure 7F). In accordance with earlier published data (Bulliard *et al.*, 2014), the reduced Treg depletion was then associated with less effective tumor control in hFcγRIIB Tg and NoTIM mice, albeit without statistical significance (Figure 7G). We reasoned that the relative lack of therapeutic impact in the face of clear differences in Treg deletion was due to the ability of the anti-mOX40 mAb (like anti-4-1BB mAb) to evoke anti-tumor effects through multiple mechanisms. We have previously shown that antibodies to both of these targets can elicit anti-tumor responses through either depletion of Tregs (requiring efficient engagement of activating mFcγRs) or direct co-stimulation on effector T cells (in part dependent upon the presence of the inhibitory mFcγRII) (Buchan et al., 2018; Griffiths *et al.*, 2020), with the therapeutic impact the net result from these activities.

Therefore, we returned to consider the therapeutic impact of the NoTIM receptor on a *bone fide* direct targeting mAb in a malignant B cell model lacking these complexities. We chose the Eμ-TCL1 lymphoma model (Bichi et al., 2002), previously used by us to assess the depletion capabilities of anti-mCD20 mAb (Carter et al., 2017; Oldham et al., 2020; Tipton *et al.*, 2015b). By inoculating mFcγRII$^{-/-}$, hFcγRIIB Tg and NoTIM mice with mCD20-expressing Eμ-TCL1 lymphoma cells, we were able to evaluate the concurrent anti-mCD20 mAb-mediated depletion of malignant and normal B cells from the blood as well as monitor tumor control (Figure 8A-B). Following tumor engraftment and detection in the blood, groups were randomized to receive anti-mCD20 mAb mIgG2a or isotype control. Mice were then bled after 2 days, 7 days and thereafter weekly until the experimental endpoint was reached. Tumor cells were identified as CD19^{+}CD5^{+}B220^{lo} by flow cytometry, alongside normal B cells (CD5^{-}B220^{hi}). Relative depletion was assessed after 2 weeks (Figure 8C) and then leukemia burden over time, in addition to long-term survival (Figure 8D-E). Growth of the Eμ-TCL1 cells in mFcγRII$^{-/-}$, hFcγRIIB Tg and NoTIM mice was equivalent following treatment with the isotype control, whereas, significant depletion resistance was observed in the NoTIM and to a lesser extent hFcγRIIB Tg mice (Figure 8C-D). This resistance was translated to a more rapid repopulation and expansion of the tumor cells after treatment, resulting in a shorter median time of survival (42 days for mFcγRII$^{-/-}$ versus 28 days for NoTIM mice; Figure 8E). Normal B cells also recovered much more rapidly in NoTIM versus mFcγRII$^{-/-}$ mice (Figure S5). Together these data clearly demonstrate that a non-signaling hFcγRIIB receptor (NoTIM) can strongly impair target cell depletion and compromise therapeutic efficacy.

Discussion

Therapeutic mAb remain the most exciting class of new drugs, with over 100 now approved for the treatment of human disease and more being approved each year (Kaplon et al., 2022). Although checkpoint blocking and immune-stimulatory mAb are currently receiving much attention, it is salient

that >40% of approved therapeutic antibodies function through direct targeting of diseased cells, such as malignant or autoimmune B cells in the case of rituximab, and breast or colorectal cancer cells in the case of trastuzumab and cetuximab. Therapeutic efficacy of these mAb relies at least in part on activating FcγRs and is impaired through expression of the inhibitory FcγR, FcγRIIB (Clynes *et al.*, 2000). We previously showed that blockade of hFcγRIIB using highly specific mAb enhanced anti-CD20 mAb mediated depletion of both normal and malignant B cells *in vivo* (Roghanian *et al.*, 2015) with the resulting combination currently being explored in the clinic (ClinicalTrials.gov registration numbers: NCT03571568; NCT04219254).

In the current study, we explored the functions of hFcγRIIB that impair target cell depletion and which format of mAb-mediated hFcγRIIB blockade was most effective to overcome them. Using a variety of model systems in which the target and/or effector cells express hFcγRIIB, we demonstrate that a WT Fc-functional anti-hFcγRIIB mAb provides optimal effects in systems where hFcγRIIB is expressed solely on the target (*e.g.*, hFcγRIIB^{+} B cells), both in monotherapy and combination with hCD20 mAb. Similar effects were also shown recently by Lu *et al*. (Lu et al., 2020), with the Fc-null (N297A) format failing to elicit direct cytotoxicity *in vitro* or efficacy *in vivo* in a variety of xenograft models. In contrast Fc-enhanced formats demonstrated more potent effects. However, it is important to note that these mAb were explored in contexts that lacked hFcγRIIB expression on the host effector cells. In the current study, we also assessed the impact of blocking hFcγRIIB in systems where hFcγRIIB was present on the effector cells but lacking from the target (hFcγRIIB^{-} B cells or Tregs), reflective of most tumors outside of B cell malignancies. Here, an Fc-null hFcγRIIB mAb was most effective at augmenting target cell depletion in combination with therapeutic mAb, with Fc-functional anti-hFcγRIIB proving detrimental. In dissecting the molecular basis for the observed preference for functional WT or Fc-null domains, we showed that hFcγRIIB ITIM phosphorylation was triggered by the former but not the latter in myeloid effector cells, initially supporting that hFcγRIIB mediates its inhibitory effects by ameliorating downstream signaling (Bruhns and Jonsson, 2015). However, our subsequent studies showed this not to be the case.

Generating and validating a non-signaling, ITIM-mutated hFcγRIIB Tg (NoTIM) mouse model, we showed that ITIM signaling was dispensable for mediating hFcγRIIB inhibition of target cell depletion. Strikingly when mCD20 mAb were administered *in vivo*, the NoTIM mice were more resistant to peripheral and tissue-resident B cell depletion than signaling competent hFcγRIIB Tg or WT C57BL/6J mice. Resistance to depletion in the NoTIM was seen with both moderate (mIgG1) and strong (mIgG2a) depleting isotypes (Nimmerjahn and Ravetch, 2005). Importantly, hFcγRIIB mediated internalization of the anti-mCD20 mAb 18B12 from the surface of the target B cells was not different between mice expressing mFcγRII or hFcγRIIB. Additionally, the maintenance of the anti-mCD20 mAb

in the serum was similar; indicating that neither activity is responsible for the resistance seen in the NoTIM mice. This was expected, given our previous demonstration that mAb:CD20:hFcγRIIB internalization appears linked to physical distortion of the plasma membrane and independent of hFcγRIIB signaling (Vaughan *et al.*, 2015). Nevertheless, the relatively low level of internalization of mCD20 and anti-mCD20 mAb observed on both WT B cells and those expressing hFcγRIIB, was initially surprising, given the rapid internalization of rituximab and hCD20 on normal B cells and B cell lymphomas (Lim *et al.*, 2011; Vaughan *et al.*, 2014). This low level of internalization is, however, fully in keeping with that seen with Type II anti-CD20 mAb such as obinutuzumab and indicates that 18B12 is a Type II mAb (Lim *et al.*, 2011; Vaughan *et al.*, 2014) (further supported by our preliminary data showing that it is not effectively redistributed into Triton-X100 insoluble lipid rafts (data not shown)).Using adoptive transfer models, we showed that expression of a non-signaling hFcγRIIB on effector cells, not target B cells, was responsible for the inhibition of mAb depletion. Inhibition was most likely achieved through competition between activating and inhibitory FcγRs for therapeutic mAb Fc. Moreover, anti-mCD25 and -mOX40 mAb-mediated depletion of Tregs highlighted that this phenomenon is not CD20 or B cell-specific. Treg depletion was also blunted in the NoTIM as compared to mFcγRII$^{-/-}$ mice and more prominent than in hFcγRIIB Tg mice. Therefore, hFcγRIIB negatively regulates direct targeting mAb in an ITIM signaling independent manner.

During our studies we also explored means through which to optimally overcome hFcγRIIB. When target cells expressing the NoTIM receptor were transferred into mFcγRII$^{-/-}$ mice, an Fc-null anti-hFcγRIIB mAb was shown to have no inherent depleting activity and did not significantly improve rituximab-mediated depletion. In contrast, an Fc-WT anti-hFcγRIIB mAb induced potent depletion as monotherapy and alongside rituximab – demonstrating that ITIM signaling is not required when hFcγRIIB is operating as a B cell target and that targeting hFcγRIIB *per se* is not an issue for effective depletion.

In parallel experiments where the NoTIM was present on the effectors and not target cells, an Fc-functional hFcγRIIB blocking mAb was shown not to improve hCD20 mAb-mediated depletion in the NoTIM mice, whereas, an Fc-null hFcγRIIB blocking mAb did, providing depletion equivalent to that observed in mFcγRII$^{-/-}$ mice. In the absence of alternative explanations (accelerated loss of mAb from the serum, removal from the cell surface or retained residual inhibitory signaling capacity) and the knowledge that surface presence was correlated with activity, we explored receptor competition as the underlying mechanism. Occupancy data in the presence and absence of WT or Fc-null anti-hFcγRIIB mAb showed that the WT Fc was able to occupy the Fc-binding region of activating mFcγRs - namely mFcγRIV with a significant effect seen on macrophages – thereby reducing rituximab-FcγR interactions and depletion. mFcγRIV (along with mFcγRI) has been shown to be critical for mediating

depletion of target cells (Gul *et al.*, 2014), therefore the significant effect seen on mFcγRIV in our data explains how WT hFcγRIIB mAb could reduce target cell depletion. mFcγR occupancy levels confirmed that the Fc-null hFcγRIIB mAb efficiently blocked hFcγRIIB without interaction with activating mFcγRs, increasing the probability of anti-hCD20 mAb:activating mFcγR interactions and therefore depletion. This behavior has been reported previously, variously termed the *Scorpion* or Kurlander effect (Kurlander, 1980; 1983), and was described by us and others in examining expression and blockade of FcγR (Hamaguchi et al., 2006; Tipton et al., 2015a). Our experiments targeting hCD40 when expressed solely on the target cells or concurrently on the effector cells indicate the broad generality of our findings, demonstrating that this same competition for FcγRs can result in thwarted target cell depletion, when targeting antigens other than CD20 and hFcγRIIB.

To understand if this finding applied to other targets and cell types, and in a therapeutic context, anti-mCD25-and anti-mOX40 mediated depletion of Tregs was assessed. As before, NoTIM mice were resistant to mAb-mediated depletion of target cells. To target mCD25, we used PC61 rIgG1 that binds mFcγRIII as an activating FcγR (Arce Vargas *et al.*, 2017), suggesting competition between hFcγRIIB and mFcγRIII for the Fc. This is in agreement with our data from targeting CD20, which shows that competition between activating and inhibitory FcγRs drives reduced target cell depletion. They are also concordant with evidence that removal of mFcγRII$^{-/-}$ or selection of mAb isotypes with higher A:I ratio elicit more powerful Treg depletion (Arce Vargas *et al.*, 2017). For targeting mOX40, we employed the more potent mIgG2a isotype of anti-mOX40 and showed similar inhibition of Treg depletion in the NoTIM mice. Furthermore, we were able to demonstrate that the lack of depletion rendered the mice more resistant to anti-OX40 mAb therapy seen with the EG7 thymoma. Finally, we returned to assess the ability of anti-mCD20 mAb to deplete malignant B cells in the various mouse strains, showing that once again expression of the NoTIM receptor impaired target cell depletion, in this case of Eμ-TCL1 tumor cells. Less effective depletion led to more rapid tumor repopulation and shorter median survival compared to mice lacking the receptor.

These results were initially surprising as a substantial body of work suggests ITIM signaling is central to the inhibitory activity of hFcγRIIB and other inhibitory receptors (Getahun and Cambier, 2015; Muta *et al.*, 1994; Stopforth et al., 2016). However, when hFcγRIIB is assessed in relation to other ITIM containing immunoreceptors, on reflection it seems unlikely to deliver its regulatory function through ITIM signaling alone. hFcγRIIB possesses a single ITIM whereas most inhibitory receptors contain two or more ITIMs in their intracellular tails, with inhibitory leukocyte Ig-like receptors (LILR) ranging from 2-4 ITIMs (De Louche and Roghanian, 2022; van der Touw et al., 2017), SIRPα 3 ITIMs (Zen et al., 2013), and LAIR1 2 ITIMs (Kang et al., 2015). Furthermore, there are usually multiple inhibitory receptors capable of regulating multiple activating receptors, such as within the KIR receptor system (Rajalingam, 2012).

In addition, ITIM containing inhibitory receptors largely bind to ligands without competition (*e.g.*, SIRPα and CD47) or through interactions between activating and inhibitory receptors which have very different ligands (KIR and LILR families) allowing for effective transduction of inhibitory regulation. In contrast, hFcγRIIB, as the sole inhibitory IgG receptor of the FcγR family and with a single ITIM, does not have any of the additional advantages of other inhibitory receptors that allow potent regulatory signaling to ensue. Taken together, it seems unlikely that hFcγRIIB ITIM-mediated signaling would be able to singularly regulate the diverse activities of multiple ITAM-containing activating FcγRs. In this context, additional regulation through the competition for Fc, as described here, would afford the greater inhibitory capacity. Although not studied here, and not relevant for the effector cells, the ITIM containing Fc receptor-like (FCRL) family of receptors, such as FCRL2-6, may contribute more widely to Fc-mediated outcomes on B cells in humans (Li et al., 2014). Furthermore, it is also worth noting that alternative means of inhibiting activating FcγRs exist, such as the ITAMi pathway, evoked following sub-optimal receptor engagement (Aloulou et al., 2012).

Overall, our study demonstrates that inhibition of target cell depletion by direct targeting therapeutic mAb is mediated by hFcγRIIB on effector cells through competition between activating FcγRs and hFcγRIIB for the Fc of the therapeutic mAb in an ITIM signaling-independent manner. This inhibition of myeloid effector cells can be overcome through use of an appropriately engineered (Fc-null) anti-hFcγRIIB mAb, restoring engagement of activating FcγRs of the Fc of the therapeutic mAb.

Limitations of the study

This study shows how FcγRIIB inhibits direct targeting antibodies independently of ITIM signaling with evidence suggesting that competition with activating FcγRs is the main mechanism of action. One limitation of our study was that it employs mice transgenic for just a single human FcγR, FcγRIIB. It therefore evaluates the impact of FcγRIIB on murine activating FcγR, not human activating FcγR. Although both species appear to exhibit broadly equivalent systems of FcγR-mediated depletion, it remains possible that differences exist. In addition, the expression level of FcγRIIB was not identical in all hFcγRIIB Tg and NoTIM mice. Although the variable level of hFcγRIIB expressing in the hFcγRIIB Tg mice was useful in helping demonstrate expression:depletion relationships (Figure S2), it would have been ideal to have equivalent expression in both strains.

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Author Contributions

A.P.S. designed and performed the experiments, analyzed, and interpreted data, and wrote the manuscript. A.R. designed and performed the experiments, supervised data collection, analyzed and interpreted data, and edited the manuscript. R.O., H.T.C., K.L.C., Y.B., S.J. and L.N.D. designed and performed certain experiments. C.P, J.K, T.I, I.M. and A.T. generated or provided key reagents or performed and analyzed the research. D.R. and P.M. helped design the study, supervised data collection, discussed and interpreted data. I.T. helped design the study, supervised data collection, discussed and interpreted data. B.F., S.A.B. and M.S.C. designed the study, supervised data collection, discussed and interpreted data and wrote the manuscript. B.F, S.A.B and M.S.C. are co-senior authors on the study.

Declaration of Interests

M.S.C. acts as a consultant for a number of biotech companies, being retained as a consultant for BioInvent International and has received research funding from BioInvent International, GSK, UCB, iTeos, and Roche. S.A.B. has acted as a consultant for a number of biotechs and receives institutional support for grants and patents from BioInvent. A.R. receives institutional support for grants and patents from BioInvent International. D.R. and P.M. are employees of GSK. I.T. and B.F. are employees of BioInvent International. This work is related to patent family EP3263602A1 concerning the combined use of FcγRIIB and CD20 specific antibodies as well as US 11001638 and WO2019020774-A2/A3, relating to OX40 and 4-1BB.

Figure Legends

Figure 1. Efficacy of CD20 mAb in combination with FcγRIIB blockade depends on FcγRIIB mAb format. A) Schematic of model systems used in figure B-D where hFcγRIIB is expressed only on the target cells. B) hFcγRIIB$^{+/-}$ x mFcγRII $^{-/-}$ target (T) and mFcγRII$^{-/-}$ non-target (NT) splenocytes labeled with high or low levels of CFSE respectively, were adoptively transferred into WT C57BL/6J recipient mice. Mice received Rituximab (RTX; hIgG1), WT or N297Q hFcγRIIB mAb (6G11; 6G or 6G-Q, respectively), or in combination as indicated, and splenic cells analyzed to determine the ratio of CD19^{+} target: non-target cells remaining; normalized to isotype control (iso) group given a ratio of 1.0. Data combined from at least 3 independent experiments. C) 2.5x10^{6} Raji cells were injected (i.v.) into SCID mice (n = 7 mice/group). Tumor-bearing mice were subsequently injected (i.p.) with 5 mg/kg of indicated mAb (Rituximab hIgG1 and 6G-Q) on a weekly basis starting on day 7, up to 4 times. Mice were monitored over time and sacrificed upon evidence of terminal tumor development. Survival was analyzed using a Kaplan-Meier survival curve. Statistical analysis was conducted using the Log-Rank test. D) Anti-tumor activity of RTX, 6G-Q or the combination in mice xenografted with human CLL cells (n=2 patients). Mice were treated with 1-10 mg/kg of either RTX, 6G-Q or both and % CLL cells remaining in the spleen enumerated and normalized to the proportion after treatment with isotype control. () Schematic of model systems used in F) where hFcγRIIB is expressed only on the effector cells. F) CFSE^{+} hCD20$^{+/-}$ x mFcγRII $^{-/-}$ target (T) and mFcγRII $^{-/-}$ non-target (NT) splenocytes were injected into hFcγRIIB$^{+/-}$ x mFcγRII $^{-/-}$ C57BL/6J recipient mice. Mice received 6G or 6G-Q mAb (2x 20 mg/kg) followed by RTX (0.2-2 mg/kg) and the ratio of splenic CFSE^{+} CD19^{+} cells determined, as before. Data combined from at least 2 independent experiments. G) Ability of WT but not Fc-null N297Q (NQ) hFcγRIIB specific mAb (6G or 6Q; 10 μg/ml for 15 min) to elicit hFcγRIIB ITIM phosphorylation (P-FcγRIIB) on mouse bone marrow derived macrophages. α-Tubulin and hFcγRIIB were used as loading controls, as indicated; representative blots shown. (B, D, F) Each dot depicts a result from an individual mouse, with mean ratios indicated by the horizontal line; error bar indicates SD. Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤ 0.0001.

Figure 2. Generation and characterization of a hFcγRIIB ITIM defective (NoTIM) mouse. A) Schematic representation of the transgene construct used to generate NoTIM mice. The construct contains the human FcγRIIB promoter, exons (E) 1-2, introns 2-3 and exons 3-7. B) Schematic and DNA sequencing profiles of the ITIM region in the NoTIM mouse showing changes resulting in substitution of Tyrosine (Y) for Phenylalanine (F). The red arrows indicate the point mutations confirmed by Sanger

Sequencing. C) Expression of hFcγRIIB was accessed on circulating B cells (human and mouse $CD19^+$), NK cells (human $CD56^+$, mouse $NK1.1^+$) and monocytes (Classical – $CD14^+CD16^-$ (mouse Ly6C High), non-classical – CD14-CD16+ (Mouse Ly6C Low) from NoTIM mouse blood using flow cytometry. Representative histograms from 3 independent experiments. D) Frozen sections from C57BL/6J WT, $mFc\gamma RII^{-/-}$, hFcγRIIB Tg and NoTIM mouse spleens were evaluated by immunofluorescence for expression of hFcγRIIB and mFcγRII. E) Bone marrow-derived macrophages (upper panel) or splenic B cells (lower panel) were isolated from NoTIM or hFcγRIIB Tg mice and stimulated with hFcγRIIB specific mAb (agonists or antagonists) at 10 µg/ml for 15-30 minutes before lysis. Lysates were probed for hFcγRIIB ITIM phosphorylation (P-FcγRIIB) and total hFcγRIIB. α-tubulin was used as a loading control. F) Splenic B cells isolated from $mFc\gamma RII^{-/-}$, hFcγRIIB Tg or NoTIM mice were cultured with 20 µg/ml heat aggregated IgG (ahIgG) for 1 hour. The proportion of total ahIgG remaining on the cell surface after 30 and 60 minutes was assessed by flow cytometry using AF488-labelled anti-hIgG. n = 3. Horizontal bars represent the mean + SD. G) Splenic B cells were isolated from $mFc\gamma RII^{-/-}$, hFcγRIIB Tg or NoTIM mice, labelled with Fluo-3-AM, washed and pre-incubated with an hFcγRIIB agonist or isotype control mAb for 15 minutes. Samples were then analyzed by flow cytometry before and after the addition of $F(ab')_2$ anti-IgM. The black arrow indicates the addition of $F(ab')_2$ anti-IgM, the white arrow indicates the addition of 0.6 µM ionomycin (positive control for calcium release). Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤ 0.0001. See also Fig. S1.

Figure 3. Anti-mouse CD20 mAb-mediated depletion of B cells is impaired in NoTIM mice. A) WT, $mFc\gamma RII^{-/-}$, hFcγRIIB Tg and NoTIM mice were treated with escalating doses of anti-mouse CD20 (α-mCD20 mAb) (18B12 mIgG1 or mIgG2a) and bled at regular intervals to ascertain B cell depletion kinetics; D = Day. (B) Gating strategy for identifying circulating B cells in peripheral mouse blood. B cells were identified as $CD19^+B220^+$. C and D) Composite data from the experiment outlined in A) using α-mCD20 mIgG1; the line graph indicates the average percentage depletion of B cells in each mouse strain (C) with individual time points shown in D), where each point represents a different mouse (n = 7 - 13 per group). The result of two independent experiments. Each column represents the mean (+ SD). Statistical analyses were conducted using a Kruskal-Wallis test with Dunn's multiple comparisons test. E-F) as C-D) but with α-mCD20 mIgG2a. G) On day 31 (D31) spleens and inguinal lymph nodes (iLN) were harvested from $mFc\gamma RII^{-/-}$ and NoTIM mice and analyzed by flow cytometry to assess B cell depletion. G) shows representative flow plots and H) bar graphs indicating the mean percentage of remaining B cells in each group (+ SD). Each point represents a different mouse (n = 7-13 mice/group).

The result of two independent experiments. Statistical analyses were conducted using a one-way ANOVA with Sidak's multiple comparisons test. * P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001, **** P≤ 0.0001.

Figure 4. Impaired depletion in the NoTIM mouse is not related to differential antibody persistence. A, B and C) WT, mFcγRII $^{-/-}$, hFcγRIIB Tg or NoTIM mice were intravenously injected with 50 µg anti-mouse CD20 (α-mCD20 mAb) mIgG1 (18B12) i.v. and bled over a 96-hour window to ascertain the level of available α-mCD20 mAb in the serum and % B cells in the blood as indicated in schedule A). The result of 1-3 independent experiments (n = 6-9 mice/group). B) The available serum α-mCD20 mAb was measured over these times and represented as the mean (+SD). C) Available serum α-mCD20 mAb concentration (top panels) and B cell depletion (lower panels) were concurrently determined at 1 hour, 6 hour and 48-hour time-points. Columns represent means (+SD); dots represent results from individual mice. D) Correlation between α-mCD20 mAb serum levels at 6h or 48h and B cell depletion for the 4 different strains. See also Fig. S3. E) Data in B and C were used to determine $t_{1/2}$, $AUC_{0\text{-}t}$, V_z and CL parameters. (C) Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparison test (serum availability) and Kruskal-Wallis test with Dunn's multiple comparisons test (% B cells). * P≤ 0.05, ** P≤ 0.01, **** P≤ 0.0001.

Figure 5. NoTIM hFcγRIIB impairs depletion through expression on effector cells. A-B) NoTIM hFcγRIIB^{+} hCD20^{+} mFcγRII $^{-/-}$ target (T) and NoTIM hFcγRIIB^{+} mFcγRII$^{-/-}$ non-target (NT) splenocytes labelled with high or low levels of CFSE, respectively, were injected i.v. into mFcγRII$^{-/-}$ mice (Schematic shown in A). One day later, mice received 2mg/kg hFcγRIIB blocking antibody (6G, 6G-Q or isotype) i.p alongside 2 mg/kg of rituximab (RTX) (or isotype) i.v. On day 2, mice were analyzed for T:NT ratio in the blood, spleen, and bone marrow by flow cytometry. Lines represent mean (+SD). Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. The result of 2-3 independent experiments. C-D) Adoptive B cell transfer of mFcγRII $^{-/-}$ x CD20$^{-/+}$ target (T) and mFcγRII$^{-/-}$ non-target (NT) splenocytes labelled with high or low levels of CFSE, respectively, were injected into mFcγRII$^{-/-}$ or NoTIM mice. On day 1, mice received 20 mg/kg hFcγRIIB blocking antibody (6G, 6G-Q or isotype) i.p and a further injection on day 2, alongside 2 mg/kg of RTX (or isotype) i.v. On day 3, mice were analyzed for T:NT ratio by flow cytometry as in B). The result of 2 independent experiments. Bars represent mean (+SD). Blue: mFcγRII $^{-/-}$ recipient mice; Purple: NoTIM recipient mice. Statistical analyses were conducted using a one-way ANOVA with Sidak's multiple comparisons test. E-F) Flow cytometry was used to analyze the availability of activating mFcγRs on immune effector cells. E) These plots show the detection of mFcγRIV on splenic macrophages from each treatment

group. F) The mean fluorescence intensity of each mFcγR on splenic macrophages, Bars represent mean (+SD) from 2-3 independent experiments. Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. G) mFcγR expression on splenic macrophages as in F, from an equivalent adoptive transfer into WT hFcγRIIB Tg mice. Bars represent mean (+SD). See also Fig. S4. * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤ 0.0001

Figure 6. Concurrent expression of hCD40 on target and effector cells limits target cell depletion. (A, B) $CFSE^{+}$ $hCD40^{+/-}$ (target) and WT (non-target) splenocytes were injected into WT (as indicated in panel A) or $hCD40^{+/-}$ (hCD40 Tg) recipient mice (as indicated in panel B) on day 0. Next day, mice received anti-human CD40 (αCD40) mAb, mIgG2a or hIgG1 (chiLOB7/4; 5 mg/kg), and the following day the ratio of splenic $CFSE^{+}$ $CD19^{+}$ cells were determined, as before (C; data combined from 2-4 independent experiments, each point represents a single mouse (n = 6-10 mice/group). Data for results in WT mice are indicated by filled symbols; left unshaded panel or in $hCD40^{+/-}$ (hCD40 Tg) mice by open symbols; right grey panel. D) Serum was collected 1 day after injection of αCD40 mAb (hIgG1) and quantified by ELISA (n = 3-6 mice/group). E-F) The expression of indicated activating and inhibitory FcγRs were assessed on splenic F4/80+ macrophages in (E) hCD40 Tg and (F) WT mice (n = 5 mice/group). Bars represent mean (+SD). * P≤0.05, ** P≤0.01.

Figure 7: mAb-mediated depletion of regulatory T cells is impaired in NoTIM mice. A, B) mFcγRII $^{-/-}$, hFcγRIIB Tg or NoTIM mice were treated with 250 µg anti-mCD25 (α-mCD25) mAb (PC61 rIgG1) i.p on Day 0 and were bled at day 2 and day 4 to analyze the kinetics of regulatory T cells (Treg) depletion by flow cytometry, with the proportion of Treg in the spleen assessed on day 7. A), example flow cytometry plots; Tregs were identified based on expression of CD4 and FOXP3. B) % Treg in each mouse group compared to isotype mAb treated group plotted as % CD4+FOXP3+ cells. The result of 3 independent experiments. Line represents the mean (+SD). Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. Significance under the data plots is treatment group compared to irrelevant. Significance above the data plots represents significance between treatment groups. C, D) Mice were injected with $0.5x10^{6}$ EG7 cells s.c., then once tumors were established mice were treated with 200 µg anti-mOX40 (α-mOX40) mAb (OX86 mIgG2a) or isotype control and then again two days later. Changes in Tregs within the periphery were analyzed on day 2 and day 4 with the change in % CD4+FOXP3+ and CD8:Treg ratio shown. The result of 3 independent experiments. Statistical analyses were conducted using a two-way ANOVA with Tukey's multiple comparisons test. E, F) the same changes were assessed in spleen (E) and tumor (F) on day 4

(D4). The result of 2 independent experiments. Line represents the mean (+SD) Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. F) The change in % CD4+FOXP3+ on D4 in the tumor. The result of 2 independent experiments combined. Line represents the mean (+SD). Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. G) Kaplan-Meier survival in E.G7 tumor bearing mice treated with α-mOX40 mAb or isotype control. Mice were either treated with 200μg α-mOX40 mIgG2a or isotype control on day 0 and again on day 2 and monitored until terminal endpoint. Statistical analyses conducted using the Log-Rank test. Statistical analyses next to isotype denotes significance between isotype treated and αOX40 mAb treated mice (e.g., NoTIM isotype and NoTIM treated). * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤ 0.0001

Figure 8: mAb-mediated depletion of malignant B cells is impaired in NoTIM mice, compromising therapy. A) mFcγRII $^{-/-}$, hFcγRIIB Tg or NoTIM mice were injected with 5x10^{6} Eμ-TCL1 cells i.p and monitored for tumor growth. When tumor equated to 10-20% of peripheral lymphocytes, mice were treated with 100 μg anti-mCD20 (α-mCD20) mAb (18B12 mIgG2a) or isotype control. B) Flow cytometry was used to assess depletion of both tumor (CD19+/B220lo/CD5+), and normal B cells (CD19+/B200hi/CD5-); plots show depletion after 2 days. C) Tumor cells and B cells were quantified on day 14 and normalized to the number of cells before treatment to plot percentage change from baseline. Line = mean. The result of 3 independent experiments (n = 8-9 mice/group), statistical analysis was conducted using a one-way ANOVA with Tukey's multiple comparison test. D) The change in the proportion of tumor cells within the periphery following treatment were quantified by flow cytometry over time. E) Survival was analyzed using a Kaplan-Meier survival curve. Statistical analysis was conducted using the Log-Rank test. Significance between isotype and α-mCD20 mAb treated mice is denoted by the line between groups, whilst significance between treatment groups is defined by parenthesis between those treated with α-mCD20 mAb. * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤ 0.0001.

Supplemental data and Figures

Supplementary Figures

Figure S1, related to Figure 2. Characterization of the hFcγRIIB NoTIM mouse. A) The presence of the NoTIM transgene was assessed by PCR amplification from genomic DNA using gene-specific primers to identify positive and negative progeny. The transgene included the 400bp region upstream of the human start codon to foster human tissue-specific gene expression. B) Flow cytometry was then used

to confirm the cell surface expression of the receptor. Grey: isotype; blue: negative sample; purple: NoTIM sample. Representative histogram gated on $B220^{+}CD19^{+}$ cells. C) Flow cytometric gating strategy used to identify human B cells, NK cells, classical and non-classical monocytes (MO) as used in Fig. 2C. D) Flow cytometric gating strategy used to identify murine B cells, NK cells, classical and non-classical monocytes (MO) as used in Fig. 2C. E) Splenic B cells were isolated from NoTIM and hFcγRIIB Tg mice and stimulated with hFcγRIIB specific mAb at 10 µg/ml for 30 minutes and lysed. Lysates were then probed for phosphorylated SHIP-1 (P-SHIP1), total SHIP-1 and α tubulin as a loading control. F-H) To understand if the loss of the endogenous mFcγRII and the introduction of the hFcγRIIB transgene impacted on the expression of activating mFcγRs, BMDMs were differentiated from the various mouse strains and flow cytometry used to investigate cell surface expression of FcγRs. Expression of FcγR on BMDMs derived from C57BL/6J WT, mFcγRII $^{-/-}$, hFcγRIIB Tg and NoTIM mice is shown. Gating shown in (E) with representative histograms for FcγR shown in (F) and the mean fluorescence intensity (MFI) from 3 independent experiments combined in (G); grey = isotype control. Color represents the relevant FcγR. Columns represent the mean (+SD). Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. * P≤0.05, ** P≤0.01, *** P≤ 0.001, **** P≤ 0.0001.

Figure S2, related to Figure 3. Relationship between hFcγRIIB Tg expression and anti-mCD20 mAb-mediated depletion. A) The expression of hFcγRIIB on B cells from hFcγRIIB Tg mice was measured by flow cytometry prior to the anti-mCD20 (α-mCD20) mAb treatment and correlated to the % remaining B cells (as a % of pre-bleed) on day 14 after treatment from experiments shown in Fig. 4. The R^2 values were calculated for from the line of best fit (R^2 = 0.6072; p 0.0017). B) The % of hFcγRIIB positive B cells from hFcγRIIB Tg mice was measured by flow cytometry prior to α-mCD20 mAb treatment and correlated to the % remaining B cells (as a % of pre-bleed) on day 14 from experiments shown in Fig. 4 (R^2 = 0.5019; p 0.0067). C) Correlation of % positivity of the hFcγRIIB transgene between B cells and monocytes (R^2 0.7558; p <0.0001). Given the tight correlation, and ease of screening the majority of mice were screened solely for hFcγRIIB expression on B cells. D) The MFI of hFcγRIIB on splenic Ly6C high monocytes from healthy hFcγRIIB Tg mice was measured by flow cytometry and correlated with the MFI of hFcγRIIB on splenic B cells. The R^2 values were calculated for from the line of best fit (R^2 = 0.5394; p 0.0101).

Figure S3, related to Figure 4. Impairment of B cell depletion in NoTIM mice is not due to insufficient serum levels or rapid internalization of mAb. A) Titration and saturation binding curve of anti-mCD20 (α-mCD20) mAb mIgG1 binding to mouse B cells from mFcγRII$^{-/-}$, hFcγRIIB Tg and NoTIM mice;

indicating a saturation level of ~2.5 μg/ml in each case. B) Relationship between hFcγRIIB Tg expression and $t_{1/2}$ in hFcγRIIB Tg and NoTIM mice. No clear correlation was observed, indicating that FcγRIIB expression does not influence α-mCD20 mAb serum persistence. C, D and E) Splenic B cells were isolated from WT, mFcγRII$^{-/-}$, hFcγRIIB Tg and NoTIM mice and incubated in vitro with anti-mCD20-Alexa 488 mIgG1 (18B12) (5 μg/mL) for 2, 6 or 24 hours. Cells were then washed and then incubated in the presence or absence of anti-Alexa 488 quenching mAb as indicated in the schematic (C). D) indicates representative data after 0 and 24h. The fluorescence remaining after quenching indicates the proportion of internalized mAb, with % internalization calculated, and results plotted from 3 independent experiments in E) (conducted with triplicate samples). Columns represent means (+SD). Red: C57BL/6J WT; mFcγRII$^{-/-}$: blue; hFcγRIIB Tg: green; and NoTIM: purple. The data show that the presence of any FcγRIIB (mouse or human, WT or NoTIM) increases internalization but that this does not differ between the different FcγRIIB molecules.

Figure S4, related to Figure 5. NoTIM hFcγRIIB impairs depletion through expression on effector cells not target cells. A) NoTIM hFcγRIIB^{+} hCD20^{+} mFcγRII$^{-/-}$ target (T) and NoTIM hFcγRIIB^{+} mFcγRII$^{-/-}$ hCD20^{-} non-target (NT) splenocytes labelled with high or low levels of CFSE, respectively, were injected i.v. into mFcγRII$^{-/-}$ mice (as detailed in Fig. 5). One day later, mice received 2 mg/kg hFcγRIIB blocking antibody (6G, 6G-Q or isotype) i.p alongside 2 mg/kg of rituximab (RTX) (or isotype) i.v. On day 2, spleens were taken and assessed for the expression of FcγRs at the surface of F480^{+} myeloid effector cells. The upper panel represents the schema, with data representing depletion of target cells within the bone marrow showing mean (+SD) from 2 independent experiments below. Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. B) mFcγRII$^{-/-}$ x CD20 Tg target (T) and mFcγRII$^{-/-}$ non-target (NT) splenocytes labelled with high or low levels of CFSE, respectively, were injected into NoTIM mice. On day 1, mice received 20mg/kg hFcγRIIB blocking antibody (6G, 6G-Q or isotype) i.p and a further injection on day 2, alongside 2 mg/kg of RTX (or isotype) i.v. On day 3, spleens were taken and assessed for the expression of FcγRs at the surface of myeloid cells. Above represents the experimental schema with the depletion of target cells within the bone marrow below. Statistical analyses were conducted using a one-way ANOVA with Sidak's multiple comparisons test. C) represents summed expression of FcγRs on splenic Ly6C+Ly6G+ cells. D) is the summed data of expression on Ly6C High cells. Bar = mean (+SD) from 2-3 independent experiments. Statistical analyses were conducted using a one-way ANOVA with Tukey's multiple comparisons test. * P≤0.05, *** P≤ 0.001, **** P≤ 0.0001. In summary detection of mFcγRIII and IV on both Ly6G+Ly6C+ and Ly6C^{hi} cells was reduced by the presence of 6G (in the presence or absence

of RTX) but not by 6G-Q (in the presence or absence of RTX) when the NoTIM is expressed only on the effector cells and not the targets.

Figure S5, related to Figure 8. NoTIM hFcγRIIB impairs depletion of B cells in the Eu-TCL1 model. mFcγRII$^{-/-}$, hFcγRIIB Tg or NoTIM mice were injected with 5x10^{6} Eμ-TCL1 cells and monitored for tumor growth. When tumor equated to 10-20% of peripheral lymphocytes, mice were treated with 100 μg anti-mCD20 (α-mCD20) mAb (18B12 mIgG2a) or isotype control. Flow cytometry was used to assess depletion of both normal B cells (CD19^{+} B200^{hi} CD5^{-}); plotted is the kinetics of B cell depletion in each mouse model following treatment with mAb. Line = mean. The result of 3 independent experiments (n = 8-9 mice per group), The data shows that normal B cells are effectively deleted from the periphery in the presence of the Eμ-TCL1 cells at day 2 in all mice but that the B cells in the NoTIM mice recover more rapidly back to normal levels compared to mFcγRII$^{-/-}$ mice with hFcγRIIB Tg mice intermediate.

Methods

STAR Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Professor Mark Cragg (msc@soton.ac.uk)

Materials Availability

All unique/stable reagents generated in this study are available, where not constrained with third party agreements, from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

Data supporting the current study are available from the corresponding author upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Mouse (m) FcγRII $^{-/-}$ mice and human (h)CD20 Tg mice have been described previously (Beers et al., 2008). hCD40 Tg mice have been previously described (White et al., 2015). hFcγRIIB$^{-/+}$ mice have been described previously (Roghanian et al., 2015). For NoTIM $^{+/-}$ mice, the ITIM Y273F and Y254F mutation was generated using site-directed mutagenesis from the full length FCGR2B2 coding region amplified from the human Burkitt's lymphoma Raji cell cDNA and introduced into the mouse genome through microinjection of C57BL/6J oocytes by Cyagen. NoTIM $^{+/-}$ and hFcγRIIB$^{-/+}$ mice were intercrossed with mFcγRII$^{-/-}$ mice (C57BL/6J) to remove the endogenous mouse inhibitory receptor. NoTIM progeny were screened by PCR (amplifying genomic DNA extracted from ear tips) or flow cytometry of peripheral blood. NoTIM$^{+/-}$ mFcγRII$^{-/-}$ mice were crossed with hCD20 transgenic (Tg) mice to generate hCD20^{+} x NoTIM$^{+/-}$ x mFcγRII$^{-/-}$ progeny. C57BL/6J and BALB/c mice were purchased from Charles River and then bred and maintained in local animal facilities, alongside other strains, in accordance with the UK Home Office guidelines. All experiments were conducted under UK Home Office licenses PPL30/1269 and P4D9C89EA following approval by local ethical committees, reporting to the Home Office Animal Welfare Ethical Review Board (AWERB) at the University of Southampton and were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. Experiments used both male and female mice and mice were

age and sex matched within experiments. For the majority of experiments mice were aged between 16-24 weeks. Littermates of the same sex were randomly assigned to experimental groups at the start of the experiment. For the majority of experiments mice were maintained in SPF conditions in IVC caging. Food (irradiated RM1 (E)) and water was available ad libutum, mice were maintained on a 12-hour light/dark cycle and environmental enrichment was provided; temperature was maintained between 20-24^{o}C. Mice were visually checked daily if adverse effects were anticipated or if mice were nearing a humane endpoint.

Human Samples

Human biological samples were sourced ethically with informed consent. Human B cells, T cells and monocytes were purified from human blood obtained from healthy donors with prior informed consent and Ethical approval from the East of Scotland Research Ethics Service, Tayside, UK.

Cell lines

πBCL1 cells were grown in culture in RPMI supplemented with 2-mercaptoethanol (50 uM), glutamine (2 mM), pyruvate (1 mM), penicillin and streptomycin (100 IU/mL), amphotericin (2 mg/mL) and 20% fetal calf serum (FCS) (Illidge et al., 2000).

METHOD DETAILS

Antibodies and Reagents

Antibodies

Rituximab (hIgG1) and cetuximab (hIgG1) were kindly provided by University Hospital Southampton Pharmacy. 6G11 (hIgG1[BI-1206] (6G), hIgG1 N297Q mutant [BI-1607] (6G-Q)) and 6G08 (hIgG1) N297Q mutant were kindly provided by BioInvent International AB. 6G11 has been previously described (Roghanian *et al.*, 2015). 18B12 (mIgG1, mIgG2a), PC61 (rIgG1), AT107-2 (rIgG1), OX86 (mIgG2a), chiLOB7/4 (mIgG2a, hIgG1) and Rituximab (mIgG1, mIgG2a) were produced in-house using stably transfected CHO-K1 cells. Purity was assessed by electrophoresis (Beckman EP; Beckman) and lack of aggregation confirmed by size exclusion (SEC) high performance liquid chromatography (HPLC). Unless otherwise stated, all antibodies were administered i.v. or i.p in 200 µL sterile PBS.

Flow Cytometry

Samples were stained with the appropriate antibody-fluorophore conjugate for 30 minutes at 4^{o}C in the dark. Samples were then washed in ACK red cell lysis buffer or Erytholyse red blood cell lysing buffer (BioRad) and subsequently washed in FACS buffer (PBS, 1% BSA, 0.01% sodium azide).

Antibodies used for staining can be found in Supplementary table 1. Samples were stored in the dark at 4°C until analysis. FACSCalibur and FACSCanto II (BD Biosciences) flow cytometers were used for data acquisition and results analyzed using FlowJo Version 10 (BD Biosciences).

Immunofluorescence

Tissues were frozen in OCT media (Cellpath) and placed in isopentane on a bed of dry ice. 10 µm frozen sections were then cut, fixed in acetone, and blocked with 5% normal goat serum before incubation with mAb to hFcγRIIB (EP888Y, Abcam), mFcγRII (AT130-5, in-house) or B cells (B220, BD Pharmingen) followed by goat anti-human-AF488 (Invitrogen), goat anti-rabbit-AF488 (Invitrogen) or goat anti-rat-AF647 (Invitrogen). Slides were mounted using Vectashield hardset with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were collected using a CKX41 inverted microscope using a Plan Achromat 10x 0.25 objective lens (Olympus). RGB images (TIFF) were transferred to Adobe Photoshop CS6 and RGB image overlays created. Background autofluorescence was removed, contrast stretched, and brightness adjusted to maximize clarity, with all images treated equivalently.

Cell isolation

Mouse splenic B cells were purified by negative selection using a MACS B cell isolation kit (Miltenyi Biotec) and collected in supplemented RPMI (RPMI 1640 containing 2 mM glutamine, 1 mM pyruvate, 100 IU/mL penicillin and streptomycin and 10% FCS (Gibco).

Generation of mouse bone marrow-derived macrophages (BMDM)

Mouse BMDMs were generated from cells isolated from the femur and tibia of mice as previously reported (Williams et al., 2013). Briefly, bone marrow cells were cultured in supplemented RPMI containing 20% L929 cell-conditioned medium (in-house). Cells were cultured for 7 days at 37°C and 5% CO_2 before use. Macrophage differentiation was confirmed by morphology and F4/80 expression.

mCD20 mIgG1 Internalization Assay

Internalization assays were performed as detailed previously (Williams et al., 2013). In brief, isolated B cells were incubated with AF488-labelled anti-mCD20 mIgG1 (18B12) (5 µg/mL) at 37°C. At stated time points, cells were washed, resuspended, and incubated at 4°C for 30 minutes in the presence or absence of anti-AF488 quenching antibody (Invitrogen) before analysis via flow cytometry. Results are presented as the percentage of internalized mAb (inversely proportional to the amount of surface accessible mAb) which was calculated as (unquenched MFI-quenched MFI)/unquenched x 100).

Calcium Flux Assay

Calcium mobilization was measured using the fluorescent probe Fluo-3-AM. Isolated splenic B cells at $1x10^7$ cells/mL were incubated with 10 µ M of Fluo-3-AM (Invitrogen) and 0.002% (v/v) Pluronic F-127 (Invitrogen) for 30 minutes at 37°C. Cells were then washed and resuspended in supplemented RPMI media for 15 minutes at 37°C. Cells were then incubated with 10 µg/mL 6G08 (hIgG1 N297Q mutation) or isotype controls for another 15 minutes at 37°C. Cells were kept warm prior to data acquisition of background fluorescence, followed by the addition of 20 µg/mL goat $F(ab')_2$ anti-mouse IgM (Jackson). Acquisition was continued for 2.5 minutes before the addition of 0.6 µ M ionomycin to elicit robust calcium flux.

Preparation of heat aggregated human IgG

Human IgG was treated at 62°C for 30 minutes to induce aggregation. The heat aggregated IgG was then separated from the monomeric fraction by size exclusion HPLC.

Heat aggregated human IgG internalization Assay

Heat-aggregated-human IgG internalization assay was performed as previously described (Vaughan *et al.*, 2015). In brief, isolated splenic B cells at $1x10^6$ cells/mL were treated with 20 µg/mL for 30 minutes at 4°C. Cells were then washed and divided into three fractions. One fraction was maintained at 4°C for 60 minutes (time zero), another fraction was maintained at 37 °C for 30 minutes and 4 °C for 30 minutes (time 30 minutes) and the last fraction was maintained at 37 °C for 60 minutes (time 60 minutes). All fractions were then stained with AF488 labelled polyclonal goat anti-human IgG (Jackson) and the MFI was quantified using flow cytometry. Internalization was expressed as the proportion of ahIgG remaining at the cell surface compared to time zero using the following formula: % cell surface ahIgG = (MFI of internalized fraction/MFI of time zero fraction) x 100.

Western Blotting

To assess the activity of the ITIM signaling pathway, $2\text{-}5x10^6$ cells isolated splenic B cells or BMDMs were washed in RPMI and cultured in supplemented RPMI before addition of irrelevant, hFcγRIIB agonist (6G08) or hFcγRIIB antagonist (6G-Q) mAb (10 µg/mL) in hIgG1 N297Q formats at 37°C for either 30 minutes (B cells) or 15 minutes (BMDMs). Cells were subsequently washed with cold PBS and lysed in Onyx buffer (containing a cocktail of protease and phosphatase inhibitors). Samples were separated by SDS PAGE on 8-10% Bis-Tris gels (Invitrogen) in MOPS running buffer. Gels were transferred to a PDVF membrane using the iBlot 2 Transfer System (Invitrogen) and blocked in 5% BSA-TBS-T for 1 hour. The membrane was then incubated with primary antibodies for 16 hours at 4°C, washed and then incubated with HRP-conjugated secondary IgG (Sigma Aldrich) for 1.5 hours at RT. ECL substrate (GE Healthcare) was used for detection and samples were imaged using the Chemi Doc-

it imaging system (UVP). Antibodies against hFcγRIIB (EP888Y, Abcam) phosphorylated hFcγRIIB (EP926Y, Abcam), SHIP-1 (2728, Cell Signaling Technology), phosphorylated SHIP1 (3941, Cell Signaling Technology) and alpha-tubulin (2144, Cell Signaling Technology) were used for Western Blotting.

Cell Binding Assay

Serum anti-mCD20 mIgG1 (18B12) titers were determined by a πBCL1 cell binding assay. Serially diluted serum samples were incubated with $0.5x10^6$ cells/mL for 15 minutes at room temperature in supplemented RPMI. Cells were then washed and stained using an anti-mouse Fc-FITC secondary antibody (Jackson) for 30 minutes at 4°C. Cells were then washed and analyzed by flow cytometry. A standard curve was generated from a known concentration of anti-mCD20 mIgG1 and used to determine anti-mCD20 IgG serum concentrations.

***In vivo* B cell depletion**

Depletion of B cells in response to escalating concentrations of the anti-mCD20 mAb 18B12 was determined using flow cytometry. In brief, tail blood was collected prior to any mAb treatment and stained using fluorescent antibodies against mouse CD19 and B220. The percentage of $CD19^+B220^+$ cells as a percentage of lymphocytes was established and normalized to 100%. Following mAb treatment, mice were tail bled according to the experimental schedule and B cells were identified as above. The percentage of $CD19^+B220^+$ cells as a percentage of lymphocytes was then normalized to the pre-bleed to ascertain the percentage of B cells remaining.

Adoptive B cell transfer assay

The relative depletion of adoptively transferred target B cells was performed as detailed previously (Beers *et al.*, 2010). In brief, $2x10^7$ splenocytes/mL were stained as target or non-target cells with 5 µM or 0.5 µM CFSE, respectively. Labelled cells were then quenched using an equal volume of FCS and washed before being combined in a 1:1 ratio and injected i.v into recipient mice (~$5\text{-}10x10^6$ cells/mouse) on Day 0. Mice were then treated with a hFcγRIIB blocking antibody (6G, 6G-Q or isotype) i.p., and i.v. with 2 mg/kg rituximab (RTX) (or isotype) or 5mg/kg hCD40 antibody (chiLOB7/4) according to the experimental schedule. Mice were then culled 16 hours following treatment with rituximab to examine the percentage of CFSE positive B cells in the blood and spleen and bone marrow using flow cytometry. Further staining of FcγRs on relevant immune effector cells was carried out at the same time.

***In vivo* regulatory T cell depletion using PC61**

Mice were treated with 250 µg of PC61 or AT107-2 (both rIgG1) i.p. on Day 0. Mice were then tail bled according to the experimental schedule to establish the percentage of peripheral regulatory T cells using flow cytometry. In brief, tail blood was stained with fluorescent antibodies against mouse CD4, CD8 and the intracellular transcription factor FOXP3. Treg cells were identified as $CD8^-$ $CD4^+$ $FOXP3^+$ and were expressed as a percentage of $CD8^-$ $CD4^+$ cells. On the final day mice were culled and Treg cells were analyzed in the blood and spleen.

***In vivo* regulatory T cell depletion using OX86 in E.G7 tumor bearing mice**

$5x10^5$ E.G7 tumors cells in 100 µL PBS were injected S.C. into the right-hand flank of mice. Tumors were measured using electronic calipers (Draper). Once tumors were established (5x5 - 7x7 mm^2) mice were treated with 2x 200 ug shots of antibody (anti-mOX40 (OX86) mIgG2a or anti-hCD20 (rituximab) mIgG2a as an isotype control) on Day 0 and Day 2 via i.p injection. Mice were then bled on Day 2 and Day 4 to ascertain Treg depletion within the periphery. Briefly, blood was analyzed by flow cytometry and based on $CD4^+$, $CD8^+$ and $FOXP3^+$ expression. T cell populations were enumerated using Precision Counting Beads (Biolegend) according to the manufacturer's instructions. Mice kept for long term survival were also bled on Day 9. Tumor size was monitored 3 times a week until experimental endpoint was reached as determined by a tumor size of 15x15 mm^2.

***In vivo* Eµ-TCL1 lymphoma depletion**

The depletion of Eµ-TCL1 cells *in vivo* was performed as previously described (Carter *et al.*, 2017). In brief, mice were given $5x10^6$ Eµ-TCL1 cells via i.p. injection. Tumor load was monitored every 7 days by assessing the percentage of Eµ-TCL1 cells in peripheral blood. In brief, mice were tail bled and were assessed for the percentage of $CD19^+CD5^+B220^{lo}$ cells as a percentage of total lymphocytes by flow cytometry. When tumor load reached 10-20% of lymphocytes, mice were treated with 100 µg antibody (anti-mCD20 mIgG2a (18B12) or anti-hCD20 (rituximab) mIgG2a as an isotype control) via i.p. injection. Mice were then bled on Day 2 and Day 7 to monitor tumor load. Mice were bled once a week from treatment until experimental endpoint was reached which was defined as two of the three following criteria being met: Eµ-TCL1 cells as a percentage of lymphocytes exceeding 80%, a white blood cell count of $>5x10^7$ cells/mL and a splenomegaly score of 3 or above (approximately 3 cm long). Eµ-TCL1 cells were monitored using flow cytometry, the white blood cell count was also monitored by flow cytometry using Precision Count Beads (Biolegend) according to the manufacturer's instructions.

Supplementary Table 1

Antigen	Fluorophore	Clone	Supplier
hFcγRII	FITC	AT 10 ($F(ab')_2$)	In-house
hFcγRIIB	AF488	6G11 (hIgG1)	BioInvent
hFcγRIII	PE	3G8 (mIgG1)	In-house
BCL-1 idiotype	FITC	MC106A5 ($F(ab')_2$)	In-house
hCD20	AF488	Rituximab (hIgG1)	UHS Pharmacy
mFcγRI	FITC	AT 152-9 ($F(ab')_2$)	In-house
mFcγRII	FITC	AT 130-2 ($F(ab')_2$)	In-house
mFcγRIII	FITC	AT 154-2 ($F(ab')_2$)	In-house
mFcγRIV	FITC	9E9 (hamster IgG)	In-house
mCD20	AF488	18B12 (mIgG1)	In-house
hCD19	APC	HIB19 (mIgG1)	Biolegend
hCD14	Pacific Blue	M5E2 (mIgG2a)	Biolegend
hCD56	APC-Cy7	5.1h11 (mIgG1)	Biolegend
F4/80	AF647	CI:A3-1 (rIgG2b N297A)	In-house
F4/80	APC	CI:A3-1 (rIgG2b)	BioRad
mCD19	PE	1D3/CD19 (rIgG2a)	Biolegend
B220	APC	RA3-6B2 (rIgG2a)	Biolegend
mCD11B	Pacific Blue	M1/70 (rIgG2b)	Biolegend
mCD4	PE	GK1.5 (rIgG2b)	Biolegend
mCD8	Pacific Blue	53-6.7 (rIgG2a)	Biolegend
mCD45.2	PE-Cy7	104 (mIgG2a)	Biolegend
mNK1.1	APC	PK136 (mIgG2a)	Biolegend

Ly6C	PerCP-Cy5.5	HK1.4 (rIgG2c)	Biolegend
Ly6G	APC-Cy7	1A8 (rIgG2a)	Biolegend
mCD5	PerCP Cy.5.	53-7.3 (rIgG2a)	Biolegend
mIgG	AF647	AB_2338861 $(Fab')_2$	Jackson Labs
hIgG	AF488	AB_2337831 (goat IgG)	Jackson Labs

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry data analysis was performed using either FCS Express software Version 3 (De Novo Software) or Flowjo Version 10.6 (BD Biosciences). All other data analysis were performed using GraphPad Prism versions 7 - 9 (GraphPad Software). Pharmacokinetic analysis was carried out using the PKSolver tool (Zhang et al., 2010). Statistical significance between two factors was analyzed using a two-tailed unpaired t-test. Statistical significance between groups was assessed by using a one-way ANOVA test unless otherwise stated. Multiple comparison tests were used as appropriate and are detailed in figure legends. The Shapiro–Wilk test was used to test for normality and determine the use of parametric or non-parametric analyses. The statistical significance in long term survival experiments was analyzed using Kaplan-Meier survival curve with the Mantel-Cox test used to assess significance between groups. Throughout, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $P \leq 0.0001$.

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