In vivo depletion using type I and II antibodies of either a mlgG1 or mlgG2a isotype. C57BL/6 mice were given a single 250 µg intravenous doses of anti-CD20 mAb. The percentage of circulating B cells compared to pre-treatment was assessed by B220 and CD19 staining over 50 days.
Supplemental Figure 2

Diminished complement activity does not affect B cell depletion. WT (n=3) or C3 knock out (C3-/-, n=3) C57BL/6 mice received ~5x10^6 splenocytes containing a 1:1 mixture of differentially CFSE stained WT or hCD20 Tg B cells. Clinically relevant antibodies were given (10 µg/ml) and the ratio of hCD20 to WT B cells remaining determined after 24 hours.
Supplemental Figure 3

Macrophage depletion blocks the ability to deplete B cells. C57BL/6 mice were treated (200 µl) with liposomes containing either PBS or macrophage depleting clodronate on days -2 and -1. Mice received ~5x10⁶ splenocytes containing a 1:1 mixture of differentially CFSE stained WT or hCD20 Tg B cells on day 0. Clinically relevant antibodies were given on day 1 (10 µg) and the ratio of hCD20 to WT B cells remaining determined after 24 hours.
Comparison of mNK cells cultured over 14 days. (A) CFSE proliferation assay, murine NK cells were isolated and CFSE stained and then cultured in vitro. The level of CFSE fluorescence was measured by flow cytometry on days 0 and 6. (B) Murine NK cells were assessed for the presence of activation markers on days 0 (black) and 14 (gray). NK cells were opsonised with 10 µg/ml mAb and analysed by flow cytometry.
Supplemental Figure 5

Human FcγR binding affinity. Target B cells were opsonised with 10×10^-3 – 10×10^-6 µg/ml human IgG1 anti-CD20 mAb and co-cultured with hFcγRIIa engineered Jurkat reporter cells (Promega). Luminescence was measured using VarioSkan plate reader.
Supplemental Methods

Animals

Mice were bred and maintained in local facilities in accordance with home office guidelines. Experiments were cleared through local ethical committees and performed under Home Office licences PPL30/2451 and PPL30/2964. Human (h) CD20 Tg mice have been described previously[1, 2]. C57BL/6 or BALB/c mice were supplied by Charles River Laboratories.

Clinical samples

Ethical approval for the use of clinical samples was obtained by the Southampton University Hospitals NHS Trust from the Southampton and South West Hampshire Research Ethics Committee (COREC 228/02/t). Informed consent was provided in accordance with the Declaration of Helsinki. CLL samples were isolated, ficoll purified and cryopreserved for subsequent analysis as detailed previously[3].

Antibodies and reagents

All mAb were produced from either the culture supernatant of hybridomas or stably transfected CHO-k1 cells. IgG was purified on Protein A with purity assessed by electrophoresis (Beckman EP system; Beckman), lack of aggregation confirmed by SEC HPLC and all preparations confirmed endotoxin low (<1 ng/mg protein) using the Endosafe-PTS system (Charles River Laboratories). Rituximab (RTX) was gifted by Southampton General Hospital oncology pharmacy. Obinutuzumab (OBZ) was from Roche. Obinutuzumab-gly (OBZ-gly; hlG1, mlG2a and m1) are non-glycoengineered versions of OBZ and were produced in-house from patent-published sequences as detailed previously[3]. Human MSCF produced in-house was amplified from IMAGE clone IRAUp969C0376D6 (Source Bioscience, UK) and cloned into pET28a (Novagen). The protein was expressed in BL21 RIPL bacteria (Agilent Technologies) and purified using a method adapted from Halenbeck et al [4]. Fluorescently conjugated mAb were purchased from Jackson ImmunoResearch, eBioscience, AbD Serotec or made in-house.

Generation of murine NK cells

Murine NK (mNK) cells were isolated from C57BL/6 mice spleens using the mNK isolation kit (Miltenyi Biotec) and maintained in complete RPMI containing mIL-2 (200 ng/ml) for 14 days.
Bone marrow derived macrophages (BMDM) were generated from the femurs of C57BL/6 mice and cultured in complete RPMI containing 20% L929 supernatant. Alternatively, human monocyte derived macrophages (hMDMs) were generated from PBMCs as previously[12, 27] and cultured in complete RPMI containing M-CSF (in-house). Target B-cells were CFSE-stained (5 µM) then opsonised with antibody before being co-cultured with macrophages for ~1h. Macrophages were stained with CD16-APC or F4/80-APC and samples assessed for the percentage of double-positive (CFSE/APC) macrophages by flow cytometry.

Detection of hIgG

The level of hIgG in mouse serum was assessed by ELISA. Briefly, maxisorp plates (Nunc) were coated with rabbit anti-hIgG (25 µg/ml) and blocked with 1% BSA, before addition of serum, incubation for 1h and washing. Rabbit anti-hIgG-HRP was added prior to incubation with OPD substrate. The OD495 nm was measured using an Epoch Microplate Spectrophotometer (BioTek) and absorbance converted to concentration using a standard curve.

Surface plasmon resonance

Surface plasmon resonance (SPR) analysis of FcγR:mAb binding was performed as described previously[28]. Briefly, antibodies were immobilised at 2000 response units [RU] to the flow cells of CM5 sensor chips (Biacore). Soluble FcγR (R&D Systems) were then injected through the flow cell at 3.125-200nM at 30 μl/min for 5min, and dissociation monitored for 10min. Affinity constants were derived by analysis of association and dissociation using a 1:1 binding model using Bioevaluation software (Biacore).

Supplemental References

### Supplemental Table 1

Affinity constants (KD) for anti-CD20 mAb binding to soluble FcγR. Anti-CD20 monoclonal antibody of either a hIgG1, mIgG2a or mIgG1 isotype were immobilised on CMS sensor chips (Biacore) and soluble mouse FcγR was passed over the chip to assess association and dissociation.

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