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Therapeutics, Targets, and Chemical Biology

Cancer Research

In vivo Cytotoxicity of Type I CD20 Antibodies Critically Depends on Fc Receptor ITAM Signaling

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Abstract

Antibody–Fc receptor (FcR) interactions play an important role in the mechanism of action of most therapeutic antibodies against cancer. Effector cell activation through FcR triggering may induce tumor cell killing via antibody-dependent cellular cytotoxicity (ADCC). Reciprocally, FcR cross-linking of antibody may lead to the induction of apoptotic signaling in tumor cells. The relative importance of these bisecting pathways to *in vivo* antibody activity is unknown. To unravel these roles, we developed a novel mouse model with normal FcR expression but in which FcR signaling was inactivated by mutation of the associated γ-chain. Transgenic mice showed similar immune complex binding compared with wild-type mice. In contrast, ADCC of cells expressing frequently used cancer targets, such as CD20, epidermal growth factor receptor, Her2, and gp75, was abrogated. Using the therapeutic CD20 antibodies ofatumumab and rituximab, we show that FcR cross-linking of antibody-antigen immune complexes in the absence of γ-chain signaling is insufficient for their therapeutic activity *in vivo*. ADCC therefore represents an essential mechanism of action for immunotherapy of lymphoid tumors. *Cancer Res; 70(8); 3209–17. ©2010 AACR.*

Introduction

Monoclonal antibodies (mAb), such as rituximab, ofatumumab, and Herceptin, are successfully used therapeutically, and many more are currently being tested in clinical trials or are in preclinical development (1). How mechanisms of action, defined by *in vitro* studies, correlate with therapeutic antibody activity *in vivo* represents an important area of research. Multiple studies in animal models and in the clinic indicate that efficient antibody–Fc receptor (FcR) interactions are crucial for antibody therapy (2–4). Antibody-FcR interactions, however, can link to multiple downstream pathways; the effect or relative importance of which is poorly understood.

FcRs bind the constant or Fc part of antibodies, are expressed on immune cells, induce phagocytosis and cellular cytotoxicity, and facilitate antigen presentation (5). Functionally, they can be grouped into activating and inhibiting FcR. Most activating FcRs lack the intrinsic capacity for signal transduction but use an associated γ-chain. The γ-chain contains an immunoreceptor tyrosine-based activation motif (ITAM) that is responsible for signal transduction. Cross-linking of FcRs leads to phosphorylation of specific tyrosines in the ITAM that in turn results in downstream signaling. Importantly, FcRs are capable of mediating antibody-dependent cellular cytotoxicity (ADCC), which is considered an important mechanism of action of therapeutic mAbs. In cancer patients, FcR polymorphisms directly affect therapeutic responses to antibodies such as rituximab (3, 6–8), and in mice lacking FcR, cancer therapeutic antibodies lose their effect on tumor growth (2, 9).

Interestingly, frequently used tumor targets, such as CD20 and Her2, have intrinsic signaling capacity that may lead to apoptosis (10, 11). Several studies using CD20 as target have shown that antibodies are more potent *in vitro* when further cross-linked by a secondary antibody (12, 13) or when tetravalent antibodies are used (14). In the case of CD20, apoptosis induction *in vitro* could also be achieved by coincubation with FcR-transfected IIA1.6 cells (12). The CD20 antibody rituximab is the most widely used antibody in the clinic against different B-cell malignancies. Antibody binding leads to clustering of CD20 molecules, which results in a change of the steady-state interaction of the associated molecules Lyn, Csk, and PAG, resulting in multiple downstream signaling events (15). In B-cell chronic lymphocytic leukemia (CLL) patients, tumor cell clearance by rituximab was associated with caspase activation and apoptosis induction (16). These findings suggest that *in vivo* cross-linking by FcR-bearing effector cells may substantially contribute to the action of therapeutic efficacy of this antibody. Importantly, the FcRγ-chain–deficient mice used to study the role of FcR lack surface expression of activating FcR, and therefore, their effector cells are also unable to cross-link tumor-bound antibodies (17).

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Consequently, it is not known to what extent apoptosis induction through cross-linking by FcR contributes to therapeutic efficacy.

To directly assess the role of *in vivo* cross-linking by endogenous FcR-expressing immune cells versus ADCC, we have generated a novel, γ-chain signaling–deficient mouse model (NOTAM). NOTAM mice exhibit normal expression of γ-chain–associated (Fc) receptors and immune complex binding but show abrogated γ-chain ITAM-dependent *ex vivo* effector functions. We show that cross-linking in the absence of FcRγ ITAM signaling is insufficient for *in vivo* therapeutic activity of CD20 therapeutic antibodies.

Materials and Methods

Plasmid construction and generation of transgenic mice. A 7.5-kb genomic clone of human chromosome 1 containing the human FcRγ-chain gene was kindly provided by J-P. Kinet (Beth Israel Deaconess Medical Center, Boston, MA). The clone consisted of 1,188 to 2,363 bp of the previously published sequence by A. Brini (Genbank L03533), containing the regulatory elements for hematopoietic-specific gene expression (18); 540 to 4,585 bp of the published sequence by H. Kuster (Genbank M33196), containing the human γ-chain gene (19); and an untranslated region (UTR) of 2,265 bp. The following primers were used to introduce the Y>F mutations (underlined): Y65F, 5′-CTTTTGTCT*CTGCA*-*G*AAATCAGATGGTGTTTTCACGG-3′ (forward) and 5′-CTGGAACTGAAAAGCTGTATCTAGTG-3′ (reverse); Y76F, 5′-GGAACCAGGAGACTTTCGAGACTCTG-3′ (forward) and 5′-CAACAC*ACTAGT*CCCTGCTCCAGCTC-3′ (reverse). Restriction sites are shown in italic. Two fragments of 645 bp (Y65F) and 564 bp (Y76F) were amplified, mixed, and used as a template for a second PCR with primers Y65F-forward and Y76F-reverse, yielding a product of 770 bp containing both mutations that was subcloned in the pGEMT vector (Promega). The Y65F/Y76F-containing fragment was excised via *Pst*I/*Spe*I and religated into the original γ-chain–containing vector (pVZ-1), after the multiple cloning site of the vector was removed by *Bam*HI/*Hin*dIII digestion. After digestion with *Kpn*I, the 7.5-kb insert containing the mutant FcRγ-chain was purified and used for microinjection into fertilized oocytes of C57BL/6 mice. Transgenic founders were identified by Southern blotting on *Bcl*I-digested samples of tail DNA using an *Nde*I fragment of human γ-chain (595 bp) as a probe. A total of four transgenic founder lines were established and crossed with γ-chain–deficient ($FcR\gamma^{-/-}$) mice (17) to obtain the NOTAM mice. Breeding of NOTAM mice followed Mendelian ratios, and none of the founder lines showed any abnormalities of the immune system. All experiments have been approved by the local animal ethical committee.

Antibodies. Rituximab (huIgG1 anti-CD20; Roche), ofatumumab (huIgG1 anti-CD20; Genmab), cetuximab [huIgG1 anti–epidermal growth factor receptor (EGFR); Merck KGaA], trastuzumab (huIgG1 anti-Her2; Roche), and F3.3 antibody supernatant (huIgG1 anti–MHC class II; kindly provided by Dr. Thomas Valerius, University Hospital Schleswig-Holstein, Kiel, Germany) were used for ADCC. Mouse IgG2a anti–glycophorin A antibody (kind gift from Dr. L. Aarden, Sanquin, Amsterdam, the Netherlands) was used for phagocytosis. The following antibodies were used for cytofluorimetry: goat polyclonal anti-FcεRI, γ-subunit (Upstate); F4/80-biotin (Serotec); FcεRI-biotin (MAR-1; eBioscience); goat anti-rabbit (H+L)-FITC (Jackson); and CD11b-APC (M1/70), CD11c-biotin (HL3), GR1-PE (RB6-8C5), FcγRII/III-PE (2.4G2), FcγRI-PE (X54-5/7.1), and CD117/c-kit-FITC (2B8; all from Pharmingen). Expression of FcγRIII was determined by blocking the cells with the FcγRII-specific antibody K9.361 followed by staining with 2.4G2. Antibodies against FcγRIV (9E9) and FcγRII (K9.361, Ly17.2-specific) were kind gifts from Drs. Falk Nimmerjahn (University of Erlangen, Erlangen, Germany) and Sjef Verbeek (Leiden University Medical Center, Leiden, the Netherlands), respectively. FACSCalibur and CantoII (BD Biosciences) were used for flow cytometry and data were analyzed using CellQuest software.

Cell culture. A431, SKBR3, B16F10, and Daudi tumor cell lines were obtained from the American Type Culture Collection and kept in RPMI 1640 (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. Mouse EL4 lymphoma cells stably transduced with human CD20 (EL4-CD20; kind gift from Dr. José Golay, Riuniti Hospital, Bergamo, Italy) were described previously (20). Bone marrow–derived polymorphonuclear cells (BM-PMN) were isolated from BM by gradient centrifugation as described (21). BM-derived dendritic cells (BMDC) and macrophages (BMDM) were cultured *in vitro* from BM in the presence of 5 ng/mL granulocyte macrophage colony-stimulating factor (CSF; Cell Sciences) as earlier described (22). Briefly, medium was refreshed on days 2 and 5. Nonadherent cells were used as BMDC and adherent cells as BMDM on days 7 to 8. BM-derived mast cells (BMMC) were cultured from BM for 4 wk in interleukin-3–supplemented complete medium. Cell cultures were routinely checked for phenotypic markers and found typically >95% pure.

Immune complex binding. OVA-IC was formed by incubating 25 μg/mL ovalbumin (OVA; grade 7; Sigma) with 100 μg/mL rabbit anti-OVA (Sigma) for 30 min at 37°C. BM-PMNs were incubated with 5 μg/mL OVA-IC at 4°C for 2 h and washed, and surface-bound OVA-IC was detected using a goat anti-rabbit IgG-FITC antibody.

Phagocytosis. BMDMs were incubated in 24-well plates with human erythrocytes opsonized with 1 or 0.5 μg/mL of anti-human glycophorin A antibody at a ratio of 1:100. Phagocytosis was allowed for 60 min at 37°C, after which bound erythrocytes were lysed with hypotonic shock. Ingested erythrocytes were counted under light microscopy.

Cell stimulation and Western blot. Microtiter plates were coated with 10 μg/mL OVA overnight and incubated with 1, 10, and 25 μg/mL of rabbit anti-OVA for 1 h. BM-PMNs were placed onto the OVA-IC for 5 min at 37°C and lysed in 20 μL of reducing sample buffer. Total lysates from 1.5×10^6 cells were subjected to SDS-PAGE and Western blotting. The following antibodies were used: phosphorylated extracellular signal-regulated kinase (ERK; clone E10; Cell Signaling) and total ERK (clone 3A7; Cell Signaling). For detection of the

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FcRγ-chain, isolated BM-PMN and cultured BMDC or BMDM were lysed, and $CD4^+$ T cells were enriched from splenocytes using a CD4-negative isolation kit (Invitrogen).

Respiratory burst. BM-PMNs were incubated with OVA-IC (1, 10, and 25 μg/mL) or 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) as a positive control for 5 h at 37°C in the presence of 500 μmol/L WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium; Serva electrophoresis] as described previously (23). Respiratory burst activity was determined with an ELISA reader (Thermo Labsystems) at 405 nm.

Tumor cell binding. EL4-CD20 cells (kind gift from Dr. José Golay) were labeled with 25 μg/mL calcein AM (Molecular Probes) for 60 min at 37°C. Labeled EL4-CD20 cells (2×10^5) with and without opsonization with 10 μg/mL of CD20 antibodies were allowed to bind to adherent 2×10^5 BMDMs in 96-well plates for 1 h at 37°C. Input fluorescent signal was determined using ELISA reader at 535 nm. Wells were washed four times with medium, and fluorescent signal was measured again. Binding is expressed as percentage with regard to input signal.

Antibody-dependent cellular cytotoxicity. A431, SKBR3, and Daudi tumor cell lines were loaded with ^{51}Cr (Amersham) and opsonized with 10 μg/mL cetuximab (huIgG1 anti-EGFR), trastuzumab (huIgG1 anti-Her2), or 40 μL of F3.3 antibody supernatant (huIgG1 anti-MHC class II, kindly provided by Dr. Thomas Valerius), respectively. Blood (25 μL) was collected from pegylated granulocyte colony-stimulating factor (G-CSF)–stimulated wild-type (WT) mice from the retroorbital plexus into Li-heparin tubes. Whole-blood cells were used as effector cells in a 200 μL reaction in RPMI 1640 + 10% FCS. Cells were incubated together for 4 h, and lysis was estimated by measuring radioactivity in the supernatant using a γ-scintillator. Percentage of specific lysis was calculated as follows: (experimental cpm − basal cpm) / (maximal cpm − basal cpm) × 100, with maximal lysis determined in the presence of 5% Triton X-100 and basal lysis in the absence of antibody and effectors. For the macrophage ADCC, BMDMs were plated together with carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled 1 μg/mL rituximab–opsonized EL4-CD20 cells at a ratio of 25:1 (250,000 BMDM with 10,000 EL4-CD20 cells). After overnight incubation, cells were trypsinized, a standard number of beads were added to each well, and the numbers of CFSE-positive cells were determined relative to 10,000 beads. Values are expressed as percentage of maximum killing/disappearance of cells, whereas 0% is typically 3,000 tumor cells (no antibody added or NOTAM cells used), and 100% would mean no recovered tumor cells.

B16F10 melanoma model. Mice were injected with 1.5×10^5 B16F10 cells i.v. On days 0, 2, 4, 7, 9, and 11, 200 μg of gp75-specific mouse IgG2a TA99 antibody were administered i.p. At day 21, mice were sacrificed and lungs were removed and scored for metastasis as previously described (24). Briefly, tumor load was defined as the sum of the following scores: metastases <1 mm were scored as 1, metastases between 1 and 2 mm were scored as 3, metastases >2 mm were scored as 10, and metastases >5 mm were scored as 50 until a maximum score of 300.

EL4-CD20 lymphoma model. Mice were injected i.p. with 5×10^6 CFSE-labeled EL4-CD20 lymphoma cells and, 16 h later, given 100 μg rituximab, ofatumumab, or 100 μL PBS i.p. The peritoneum was washed 24 h after antibody injection, and the total number of tumor cells was determined using TruCount tubes (BD Biosciences). The number of effector cells in the lavage was determined using flow cytometry. Samples were compared relative to 25,000 beads during the measurement (Interfacial Dynamics Corp.).

Statistics. Parametric data have been analyzed by unpaired *t* test, and nonparametric data with Mann-Whitney test, using GraphPad 4.0 software. A *P* value of <0.05 was considered significant.

Results

Generation and* ex vivo *characterization of NOTAM mice. FcRγ-chain–deficient mice lack the expression of activating FcR. To establish mice with normal surface FcR expression and normal immune complex binding, but with nonsignaling FcR, we generated transgenic mice harboring the human FcRγ-chain gene with inactivated ITAM (Y65F, Y76F) and the regulatory elements required for tissue-specific gene expression (Fig. 1A) and crossed them onto the $FcR\gamma^{-/-}$ background. The mouse and human γ-chain are highly homologous, with only 1–amino acid difference in the transmembrane region (Fig. 1B), the region that mediates FcR interaction. Western blot analysis showed that expression of the ITAM-deficient human γ-chain (NOTAM = no ITAM) transgene mimicked the physiologic expression pattern of the γ-chain in humans, with expression in myeloid but not in lymphoid cells (Fig. 1C). Flow cytometric analysis of NOTAM effector cell compartments showed that expression of activating FcγR (FcγRI, FcγRIII, and FcγRIV) and FcεRI was fully restored to WT levels (Fig. 1D), indicating functional association of human γ-chain with mouse FcR. Expression of the human γ-chain in transgenic BM-PMN and BMDM seemed higher than the endogenous murine γ-chain in WT PMN (Fig. 1C). However, this was not accompanied by increased FcR surface expression on these cells (Fig. 1D; data not shown). Murine FcγRII is an inhibitory FcR, which is independent of the γ-chain. Accordingly, expression of FcγRII on B cells was comparable in all strains (Fig. 1D). Binding of fluid-phase IC of OVA and rabbit anti-OVA (OVA-IC; 5 μg/mL) by NOTAM PMN was comparable with WT PMN (Fig. 2A). Binding of IC to $FcR\gamma^{-/-}$ cells was probably caused by binding to FcγRII (25).

***Abrogation of FcRγ-chain–dependent functions* ex vivo.** To confirm that ITAM-mediated activation is abrogated in NOTAM effector cells, we tested several FcR functions *ex vivo*. FcγR cross-linking on PMN induces a variety of cellular responses, including respiratory burst activity and ADCC (26). *In vitro* studies using cell lines have shown the importance of FcRγ-chain ITAM in these processes; however, this has never been studied in an endogenous system (27, 28). A downstream substrate of FcRγ-chain activation is mitogen-activated protein kinase (MAPK; ref. 29). Plate-bound OVA-IC (OVA,

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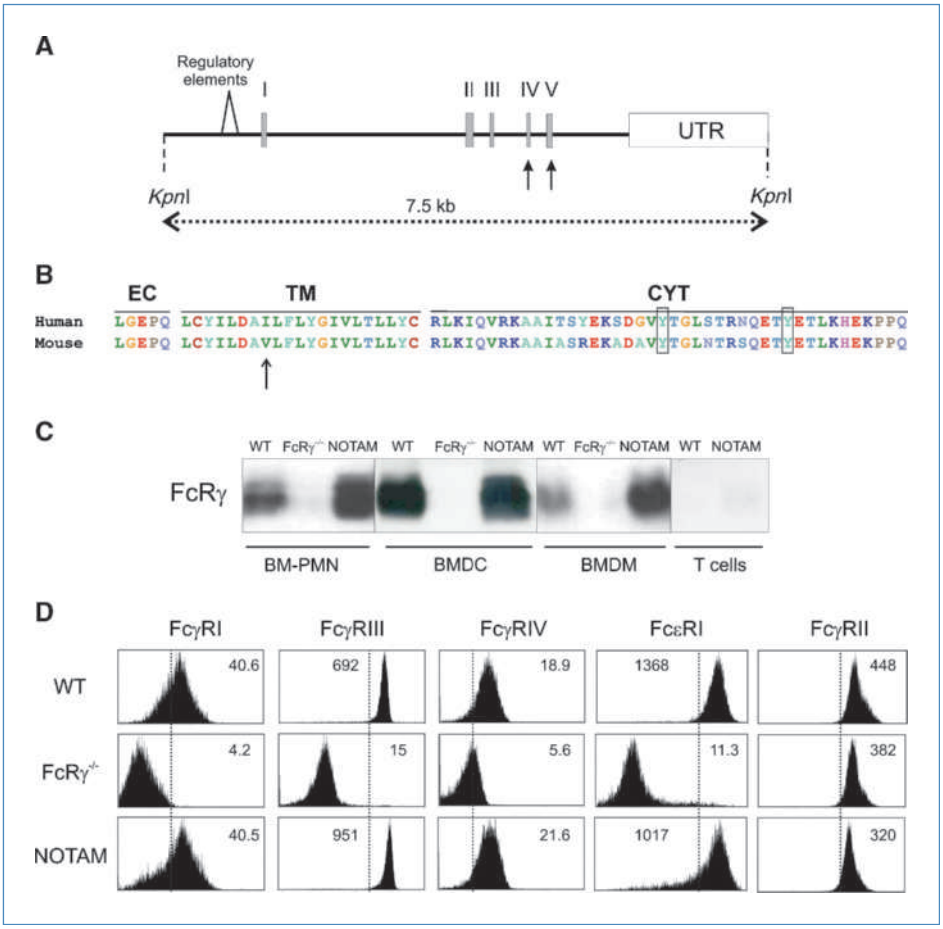

Figure 1. Generation of NOTAM transgenic mice and expression of γ-chain–associated FcR in NOTAM mice. A, schematic representation of the transgenic construct used for generation of NOTAM transgenic mice. The construct contains the regulatory elements for hematopoietic-specific gene expression, the human γ-chain gene (exons I–V), and a 5′UTR. Boxes indicate the location of the mutations that were introduced (Y65F and Y76F) as described in Materials and Methods. B, alignment of the amino acid sequence of the human and mouse FcRγ-chain. The color coding represents the type of amino acid. EC, extracellular; TM, transmembrane; CYT, cytosolic. Arrow, FcR interaction is mediated through the transmembrane region, which contains only 1–amino acid difference between mouse and human γ-chain. C, FcRγ-chain expression in whole-cell lysates of BM-PMN, BMDC, BMDM, and splenic T cells isolated from WT, FcRγ$^{-/-}$, and NOTAM mice. The antibody detects both human (NOTAM) and murine (WT) γ-chain. D, surface expression of FcR in WT, FcRγ$^{-/-}$, and NOTAM mice was determined with fluorescence-activated cell sorting (FACS) analysis. For each FcR, a representative cell type is shown (FcγRI, BMDC; FcγRIII/FcγRIV, BM-PMN; FcεRI, BMMC; FcγRII, peripheral blood B cells). Histograms and geometric means from a representative experiment (of four independent experiments) are shown.

10 μg/mL; rabbit anti-OVA, 1, 10, and 25 μg/mL) strongly enhanced the phosphorylation of p42/p44 MAPK in WT PMN after 5 minutes of incubation. In contrast, MAPK phosphorylation was absent in NOTAM PMN, showing the functional inactivation of the ITAM by the Y65F/Y76F mutations (Fig. 2B). Next, we investigated respiratory burst activity as a measure for PMN activation. We found that despite binding OVA-IC similarly to WT PMN, NOTAM PMNs *ex vivo* have a reduced capacity to release reactive oxygen species in response to stimulation with OVA-IC compared with WT PMN (Fig. 2C). NOTAM PMN reacted similarly to an FcR-independent stimulus (PMA) as WT PMN. WT BMDMs were able to phagocytose human erythrocytes opsonized with anti-human glycophorin A antibody. In contrast, FcRγ$^{-/-}$ and NOTAM macrophages were inactive in erythrocyte phagocytosis (Fig. 2D).

Taken together, despite expression of normal levels of γ-chain–associated FcRs and normal immune complex binding, FcR-mediated effector functions are abrogated in NOTAM effector cells *ex vivo*.

***NOTAM effector cells are deficient in ADCC* ex vivo.** To assess the ability of NOTAM effector cells to mediate ADCC, we used blood PMN and BMDMs, as both cell types are involved in tumor killing *in vitro* and *in vivo* (26, 30–32). Daudi B-cell lymphoma, A431 epidermoid carcinoma, and SK-BR-3 breast cancer cell lines were used as targets, opsonized with anti-HLA, anti-EGFR, and anti-Her2 antibodies, respectively. Killing of tumor cells by WT PMN was only observed in the presence of tumor-specific antibodies. Tumor cell lysis by NOTAM PMN was completely abrogated similar to findings in FcRγ$^{-/-}$ mice (Fig. 3A). In addition to natural killer (NK) cells, for CD20 antibodies, FcγR-expressing monocytes/

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macrophages were shown to play a role in B-cell depletion *in vivo* (31–33). Therefore, we assessed ADCC/phagocytosis of rituximab-opsonized EL4-CD20 cells in a macrophage-based killing assay. Similar to PMN, NOTAM-derived macrophages were unable to kill or phagocytose antibody-opsonized tumor cells (Fig. 3B). Impaired tumor killing was not due to lack of binding because BMDMs of NOTAM mice were able to bind opsonized EL4-CD20 cells *ex vivo* similar to WT mice, whereas binding of FcRγ$^{-/-}$ macrophages was markedly impaired (Fig. 3C). As all four independently generated NOTAM founder lines exhibited similar impaired ITAM signaling *ex vivo*, one representative strain was used for the following *in vivo* experiments.

Antibody tumor immunotherapy critically depends on FcRγ-chain ITAM signaling. First, we confirmed the requirement for FcR ITAM signaling in antibody therapy in the well-established B16F10 lung metastasis model. Tumor outgrowth can be prevented by the administration of mAb TA99 that targets gp75, a differentiation antigen on melanocytes. The target molecule gp75 does not have intrinsic signaling capacity (34). The importance of both activating FcR (FcγRI and FcγRIV) and effector cell populations (PMN and macrophages) was previously shown in this model (9, 24, 35). Outgrowth of lung metastases was observed in PBS-treated WT mice and was nearly absent after TA99 treatment (Fig. 4A and B). As expected, TA99 treatment did not prevent melanoma development in FcRγ$^{-/-}$ and NOTAM mice (Fig. 4A and B). Taken together, these results show the importance of FcR ITAM signaling for antibody therapy in the B16F10 model and thereby validate the NOTAM mice.

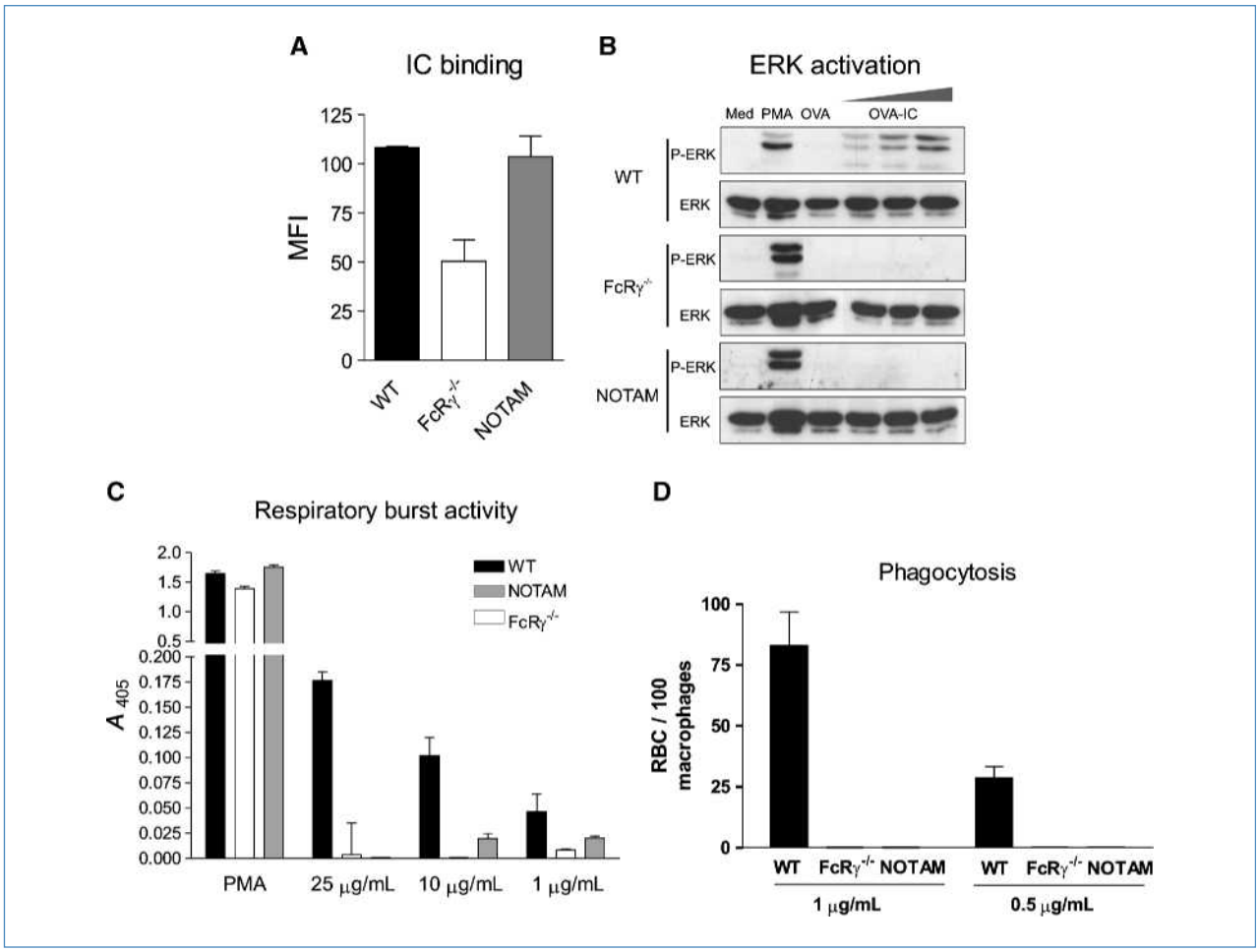

Figure 2. FcRγ-chain–dependent functions *ex vivo*. A, BM-PMNs isolated from two WT, FcRγ$^{-/-}$, and NOTAM mice were incubated with 5 μg/mL immune complexes of OVA and rabbit anti-OVA (OVA-IC) for 2 h at 4°C and washed, and bound immune complexes were detected with an anti-rabbit IgG-FITC antibody. Columns, mean of triplicates from a representative experiment (of five independent experiments); bars, SD. B, BM-PMNs were placed on immobilized OVA-IC (OVA, 10 μg/mL; rabbit anti-OVA, 1, 10, and 25 μg/mL) for 5 min at 37°C. Cells were lysed and levels of phosphorylated (P-ERK) and total ERK were determined by Western blot. PMA (100 ng/mL) was used as positive control. C, BM-PMNs were incubated with soluble OVA-IC or stimulated with 100 ng/mL PMA for 5 h. Superoxide production was determined by WST-1 reduction measured at 405 nm. Columns, mean from one representative experiment of two; bars, SD. D, BMDMs were incubated with anti-human glycophorin A–opsonized erythrocytes at 37°C for 1 h. Bound erythrocytes were lysed and ingested erythrocytes were counted under light microscopy. Columns, mean of triplicates from a representative experiment (of two independent experiments); bars, SD.

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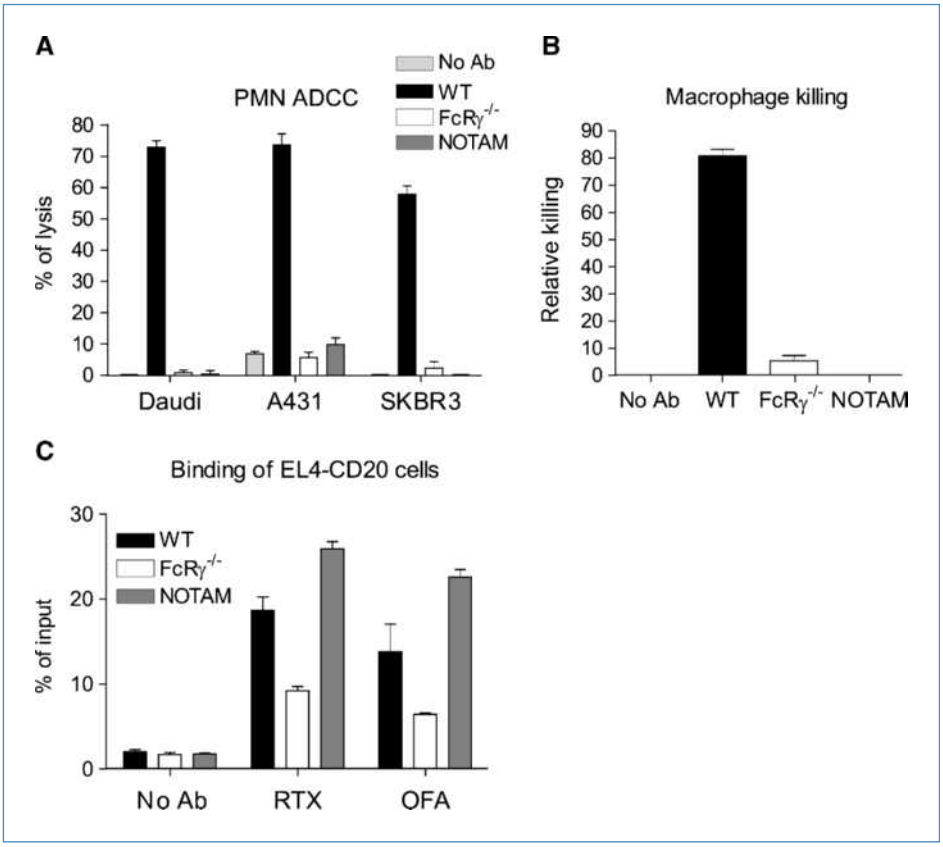

Figure 3. *Ex vivo* ADCC with NOTAM cells. A, Daudi, A431, or SKBR3 tumor cell lines were loaded with ^{51}Cr and opsonized with 40 μL F3.3 antibody supernatant (huIgG1 anti–MHC class II), 10 μg/mL cetuximab (huIgG1 anti-EGFR), or trastuzumab (huIgG1 anti-Her2) and were incubated with 25 μL whole blood from pegylated G-CSF–stimulated mice as effector cells. Cell lysis was determined by ^{51}Cr release after 4-h incubation. B, CFSE-labeled 1 μg/mL rituximab–opsonized EL4-CD20 cells were incubated overnight with BMDM at a ratio of 25:1 (BMDM to tumor cells). The numbers of CFSE-positive cells were compared between samples relative to known amount of beads. The decrease in CFSE-positive tumor cells compared with conditions without antibody is expressed as relative killing. C, calcein-labeled EL4-CD20 lymphoma cells were opsonized with 10 μg/mL rituximab (RTX) or ofatumumab (OFA) and tested for binding to a monolayer of BMDM. Data show the percentage of signal after four washing steps compared with the initial signal (input). Columns, mean of triplicates from a representative experiment (of two to five experiments); bars, SD.

***No role for cross-linking by FcR-expressing cells* in vivo.** In contrast to gp75, CD20 is a tumor target that has signaling properties. Cross-linking of anti-CD20 antibodies by a secondary antibody or by FcR-bearing accessory cells induces apoptosis in tumor cells *in vitro* (12, 36). Based on these observations, we hypothesized that FcR-induced antibody cross-linking might contribute to the therapeutic effect of CD20 antibodies *in vivo*. To test this hypothesis, we used two type I CD20 antibodies: rituximab, a chimeric antibody that was approved for cancer therapy in 1997, and ofatumumab, a recently Food and Drug Administration–approved fully human antibody that has shown clinical benefit for the treatment of refractory CLL (37, 38). Type I CD20 antibodies are able to induce apoptosis in target cells on cross-linking by a secondary antibody. We used the EL4 murine T-cell lymphoma expressing human CD20 (EL4-CD20; ref. 20). Both ofatumumab and rituximab were able to induce an increase in the number of Annexin V–positive EL4-CD20 cells when cross-linked by a secondary antibody *in vitro*, confirming the signaling capacity of CD20 on the EL4 cells (Fig. 5A). We set up a short-term intraperitoneal model using EL4-CD20 cells (20) as described in Materials and Methods. Tumor kill was determined by counting the number of tumor cells in peritoneal lavage fluid 24 hours after antibody injection. In WT mice, treatment with 100 μg rituximab and ofatumumab resulted in a significant decrease in the number of recovered tumor cells compared with PBS-treated animals, with ofatumumab being superior in tumor cell killing (Fig. 5B). Interestingly, the therapeutic effect of both rituximab and ofatumumab was completely abrogated in FcR$\gamma^{-/-}$ and NOTAM mice. Recruitment of CD11b^{+} immune effector cells to the peritoneal cavity was dependent on the presence of tumor cells but not therapeutic antibody in all strains (Fig. 5C). Taken together, these data show that FcR signaling is essential for therapy with type I CD20 antibodies in this short-term model *in vivo*.

Discussion

We have earlier shown using transfectants that mutation of the ITAM-associated tyrosines *in vitro* abrogates FcR-dependent

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phagocytosis and ADCC, but not antigen presentation (27, 28), illustrating that FcRγ-chain signaling is important, but not exclusive, for FcR function. Here, we describe and validate a novel mouse model where biological functions associated with FcR expression can be separated from those depending on signaling. A previous attempt to generate an FcRγ-chain signaling–deficient mouse using a cDNA construct resulted in reconstitution of FcR expression to only 20% of WT levels (39), rendering that model unsuitable for *in vivo* studies. In our model, γ-chain–associated FcRs are fully reconstituted (Fig. 1), whereas ITAM-dependent functions are completely abrogated *ex vivo* (Figs. 2 and 3), making NOTAM mice ideally suited for the *in vivo* study of FcR ITAM signaling and other γ-chain–associated receptor functions.

In the present study, we focused on the role of FcR signaling in antibody therapy of cancer. Therapeutic antibodies exert their antitumor effect via multiple mechanisms. These include both direct Fab-mediated mechanisms (ligand blockade, inhibition of receptor signaling, and cell cycle arrest) and indirect, Fc-mediated mechanisms [ADCC and complement-dependent cytotoxicity (CDC); ref. 40] depending on the type of antibody and the target antigen. Despite our detailed understanding of antibody effector mechanisms *in vitro*, the contribution of these mechanisms to *in vivo* (clinical) efficacy remains largely unknown. Studies in FcR-deficient mice (9, 24, 35) support a role for FcR in the antitumor activity of antibodies, either by activating immune cells or as antibody cross-linkers. Furthermore, in humans, polymorphisms in FcγRIIIa and FcγRIIa have been associated with efficacy of treatment with rituximab (CD20; refs. 3, 6), cetuximab (EGFR; ref. 7), and trastuzumab (Her2; ref. 8). Apoptosis induction by antibodies has been described for tumor targets

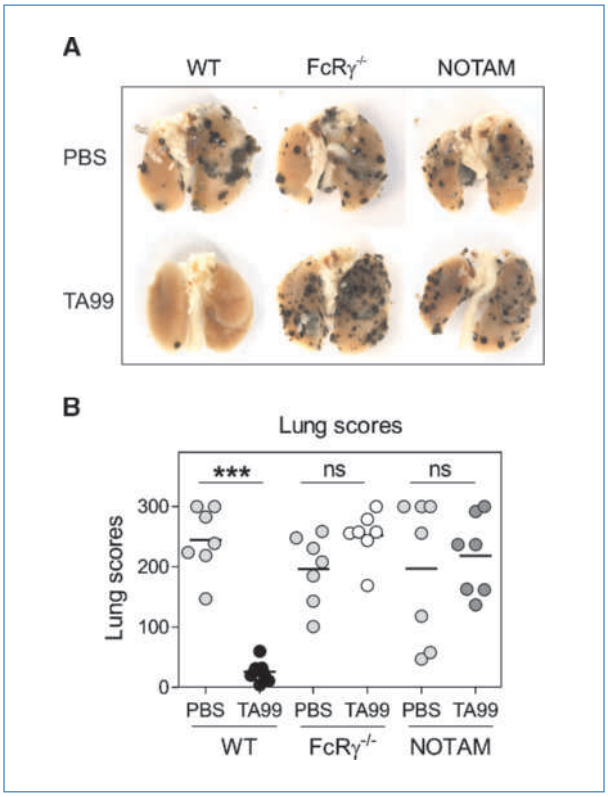

Figure 4. Antibody therapy of B16F10 melanoma requires FcRγ-chain signaling. A and B, B16F10 melanoma model. mAb TA99 (200 μg/time point) was administered on days 0, 2, 4, 7, 9, and 11 i.p. At day 21, mice were sacrificed and lungs were macroscopically scored for metastasis outgrowth (seven mice per group). ***, $P < 0.001$. One representative example of the lungs for each group of mice is shown in A. ns, not significant.

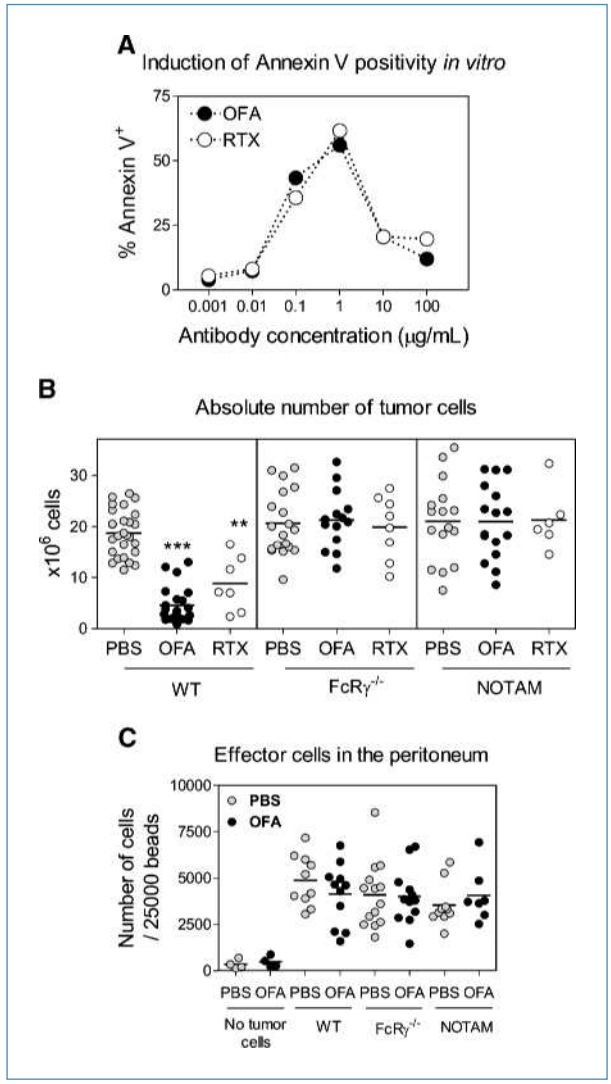

Figure 5. FcRγ-chain signaling is essential in antibody therapy of EL4-CD20 lymphoma. A, EL4-CD20 cells were incubated with varying concentrations of ofatumumab (•) or rituximab (○) in the presence of 5 μg/mL anti-human IgG antibody overnight. Percentage Annexin V^+ cells were determined by cytometry. B, mice were injected i.p. with 5×10^6 CFSE-labeled EL4-CD20 lymphoma cells and given 100 μg ofatumumab, rituximab, or 100 μL PBS i.p. 16 h later. After 24 h, the number of tumor cells in peritoneal wash fluid was determined by FACS analysis using TruCount tubes for quantification. **, $P < 0.01$; ***, $P < 0.001$. C, the number of $CD11b^+$ immune effector cells in the lavage fluid was determined by FACS analysis.

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such as CD20, CD30, and Her2 (Fig. 5A; refs. 12, 41, 42) but was never confirmed *in vivo*. In the present study, we show that FcR ITAM signaling is essential for antibody immunotherapy in two tumor models: the B16F10 melanoma model (Fig. 4) and the EL4-CD20 lymphoma model (Fig. 5).

Our data do not support a substantial role for apoptosis induced by FcR cross-linking as a mechanism of therapeutic antibodies *in vivo*. It should be noted that apoptosis induction may be dependent on the target antigen and epitope recognized and could be influenced by factors, such as FcR expression levels, antibody dose, and effector-to-cancer cell ratios. Our results indicate that under the conditions studied, with CD20 as a target molecule and 100 μg of therapeutic antibody, FcRs are only essential for ADCC. From the data presented here, we cannot exclude that a bell-shaped curve as observed *in vitro* (Fig. 5A) may occur. Similar results obtained from experiments, in which 10 times less antibody and tumor cells were used (data not shown), however, suggest that this is not evident *in vivo*.

Both CD20 antibodies used in this study were described earlier to use CDC as a major effector mechanism (20, 43). CDC was found crucial for rituximab in a nonimmunodeficient mouse model using EL4-CD20 cells (20). Because we found FcR signaling to be essential in our models, we checked for complement activity in sera from the mouse strains used in our study. Complement activity of FcR$\gamma^{-/-}$ and NOTAM serum (CH50 and *in vitro* iC3b deposition on opsonized tumor cells) were similar to WT serum (data not shown). At present, we cannot exclude a role for CDC in the current mouse model. The relative contribution of ADCC and CDC to tumor cell killing in this model is subject of further study.

Alternatively, abrogation of FcRγ may affect cell migration via impairing integrin signaling. We have seen that migration of effector cells to the peritoneal cavity was entirely dependent on the presence of the tumor (Fig. 5) and did not differ between the different mouse strains.

In general, different effector mechanisms are called on in different situations [e.g., solid tumors versus lymphoid tumors and micrometastases versus primary (large) tumors]. Therefore, it is important to generate a wide range of mAbs with distinct mechanisms of action, such as CDC, ADCC, or apoptosis induction. Alternatively, targeting distinct tumor antigens expressed on one tumor cell with combinations of antibodies seems to be another promising therapeutic improvement. In both cases, it is crucial to understand the underlying mechanism of the mAbs, and with the NOTAM mice, we provide a valuable tool for preclinical testing of the effector functions of novel therapeutic antibodies. In addition, given the FcRγ-chain association with numerous other receptors, such as PIR-A, LIR, GPVI, OSCAR, integrins, and the lectin receptors DCAR and dectin 2 (44), the NOTAM mouse opens new avenues to test a plethora of biological γ-chain ITAM-dependent functions *in vivo*, including collagen-induced platelet activation, outside-in signaling of integrins, development and function of osteoclasts, and immune functions in NK cells, neutrophils, macrophages, and dendritic cells (45).

In the present study, we used this novel mouse model to show that FcR ITAM signaling is essential for melanoma and lymphoma antibody immunotherapy.

Disclosure of Potential Conflicts of Interest

S. de Haij, F.J. Beurskens, J.G.J. van de Winkel, and P.W.H.I. Parren are Genmab employees with warrant and/or stock ownership. J.H.W. Leusen received research funding from Genmab.

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S.d.H., J.S.V., J.G.J.v.d.W., and J.H.W.L. formulated the hypothesis and initiated and organized the study. S.d.H., J.H.M.J., and P.B. performed the main experimental work and analyzed the data. J.E.B., D.L.B., and A.M. helped with some experimental procedures. J.H.W.L. oversaw the experiments and provided the main funding for the research. F.J.B. and P.W.H.I.P. contributed to data discussion. S.d.H., P.B., and J.H.W.L. prepared the final manuscript.

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In vivo Cytotoxicity of Type I CD20 Antibodies Critically Depends on Fc Receptor ITAM Signaling

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