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Complement Activation Determines the Therapeutic Activity of Rituximab In Vivo[1]

Nicola Di Gaetano,* Elena Cittera,* Rachele Nota,* Annunciata Vecchi,* Valeria Grieco,† Eugenio Scanziani,† Marina Botto,‡ Martino Introna,[2]* and Josée Golay*

Rituximab is an anti-CD20 chimeric mAb effective for the treatment of B-NHL. It can lyse lymphoma cells in vitro through both C- and Ab-dependent cellular cytotoxicity. The mechanism of action of rituximab in vivo is however still unclear. We have set up a new in vivo model in nonimmunodeficient mice by stable transduction of the human CD20 cDNA in the murine lymphoma line EL4. Animals injected i.v. with the EL4-CD20$^+$ lymphoma cells died within 30 days with evident liver, spleen, and bone marrow involvement, confirmed by immunohistochemistry and PCR analysis. A single injection of rituximab or the murine anti-CD20 Ab 1F5, given i.p. 1 day after the tumor, cured 100% of the animals. Indeed, at week 4 after tumor cell inoculation, CD20$^+$ cells were undetectable in all organs analyzed in rituximab-treated animals, as determined by immunohistochemistry and PCR. Rituximab had no direct effect on tumor growth in vitro. Depletion of either NK cells or neutrophils or both in tumor-injected animals did not affect the therapeutic activity of the drug. Similarly, rituximab was able to eradicate tumor cells in athymic nude mice, suggesting that its activity is T cell independent. In contrast, the protective activity of rituximab or the 1F5 Ab was completely abolished in syngeneic knockout animals lacking C1q, the first component of the classical pathway of C (C1qa$^{-/-}$). These data demonstrate that C activation is fundamental for rituximab therapeutic activity in vivo. ***The Journal of Immunology,*** **2003, 171: 1581–1587.**

Over the last 10 years, many humanized mAbs have been developed against different cell surface Ags expressed by neoplastic cells with the aim of obtaining novel, more specific, and less toxic anticancer agents. These molecules are either unconjugated, or conjugated to toxins, cytokines, or radionuclides that may increase their efficacy in vivo (1, 2). Despite much effort, relatively few molecules have shown sufficient clinical activity with low toxicity to be approved for the treatment of cancer. Even for clinically active molecules, there is an urgent need to enhance their efficacy. In many cases, the mechanism of action of these Abs in vivo is still little understood. Such knowledge would be of fundamental importance to develop new reagents either more active than the current generation of anticancer Abs or capable of overcoming some of the mechanisms of resistance (1).

Rituximab (Mabthera, Rituxan) is a chimeric unconjugated IgG1 mAb approved for the treatment of B-NHL and clinically active in both low grade and aggressive lymphomas (3–6). Studies in vitro have shown that rituximab is very effective in inducing C-dependent cytotoxicity (CDC)[3] against both freshly isolated lymphoma cells and cell lines (3, 7–9). Furthermore, C has been shown to be activated very rapidly by rituximab in vivo in patients (10) or in monkeys (11). Although less efficiently, rituximab also activates Ab-dependent cellular cytotoxicity (ADCC) in vitro (3, 8), and recent evidence showing a correlation between FcγRIII polymorphisms and clinical response suggests a role for FcγRIII-bearing cells such as NK cells and macrophages in the response (12). In addition, a recent model of human lymphoma in nude mice has suggested that the FcγR γ-chain, common to both FcγRI and FcγRIII, is required for the full therapeutic activity of the Ab (13). Other reports suggest a role for rituximab-induced apoptosis in the therapeutic activity of rituximab, but little evidence is available in vivo to support this hypothesis (14–16).

We have set up a nonimmunodeficient mouse model to study the mechanism of action of rituximab in vivo and show a crucial role for C activation.

Materials and Methods

Infection and selection of EL4-CD20$^+$ cells

EL4 murine T lymphoma cells and YAC-1 murine lymphoma were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% FCS (HyClone, Steril System, Logan, UT), 2 mM glutamine (Life Technologies, Paisley, Scotland), 50 μM 2-ME, and 100 IU/ml penicillin/streptomycin. EL4 cells were infected with the Moloney-derived long terminal repeat (LTR)-CD20-LTR vector containing the human CD20 cDNA, as described (17). CD20-positive cells were purified by sorting on a FACSorter (BD Biosciences, San Jose, CA). CD20 expression levels were measured using PE-labeled anti-CD20 mAb and Quantibrite beads (BD Biosciences).

Syngeneic lymphoma model

C57BL/6 mice (8–10 wk of age) were purchased from Charles River (Calco, Italy). We inoculated 8×10^3 EL4-CD20$^+$ cells in 200 μl of saline

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[3] Abbreviations used in this paper: CDC, C-dependent cytotoxicity; ADCC, Ab-dependent cellular cytotoxicity; LTR, long terminal repeat; PMN, polymorphonuclear cell.

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by tail vein injection. In parallel groups of mice, 150 μg of rituximab (Roche Italia, Monza, Italy), murine anti-CD20 IgG2a Ab 1F5 (18), or control anti-human IL-2Rβ Ab daclizumab (Roche) in 300 μl of saline, or saline only was inoculated i.p. 24 h later. All procedures with animals were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

In some experiments, groups of mice were repeatedly inoculated i.p. with anti-NK cell Ab TM-β1 recognizing the murine IL-2R β-chain or anti-GR1 Ab RB-8C5 specific for murine neutrophils, as described (19, 20). Treatment with either Ab started 2 days before tumor cell inoculation with a 300 μg dose, followed by administrations of 200 μg doses every 4 days until day 30 (19, 20). That the Abs used effectively depleted NK and polymorphonuclear cells (PMN), respectively, was verified by treating control syngeneic mice with 300 μg Ab and measuring NK activity against the YAC-1 cell line (19) or by manual counts of peripheral blood PMN after May-Grünwald-Giemsa staining, as described (20), 2 days after Ab treatment.

The C1qa$^{-/-}$ knockout mice in the C57BL/6 background have been described previously (21). They were bred at Charles River and used at 8–10 wk of age like their wild-type counterparts. In some experiments, nude mice (CD1 *nu/nu*; 8–10 wk old; Charles River) were used. Experimental conditions were the same as for the C57BL/6 mice, except that mice were irradiated (3, 5 Gy) 1 day before tumor cell inoculation. Necropsy was performed on all tumor-inoculated animals.

Immunohistochemistry of tumor

Samples from liver and spleen of rituximab-treated and untreated mice excised 28 days after tumor cell inoculation were fixed in 10% neutral buffered Formalin for 24 h and processed for embedding in paraffin blocks. Five-micron sections were cut and mounted on poly(L-lysine)-coated slides. The slides were pretreated with microwave in citrate buffer for 10 min and then immunostained using the DAKO ARK Kit (DAKO, Glostrup, Denmark). As primary Ab, the anti-CD20 7D1 clone (Novocastra, Newcastle, U.K.) was used at a 1/25 dilution. The slides were developed with 3,3-diaminobenzidine, counterstained with Mayer hematoxylin, dehydrated through graded alcohols, clarified in xylene, and mounted in the EUKIT Balsam (Bioptica, Milano, Italy).

PCR analysis

We collected whole livers, spleens, and bone marrow cells from pairs of rituximab-treated and untreated mice at days 14, 21, and 28 after tumor inoculation. The tissues were homogenized, and genomic DNA was immediately purified according to standard SDS/proteinase K extraction procedures. A total of 500 ng of genomic DNA was amplified by PCR in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.2 mM dNTP, 2 mM $MgCl_2$ with 0.5 U *Taq* DNA polymerase and 1.5×10^{-7} M of forward (5′-AATTCAG TAAATGGGACTTTCCCG-3′) and reverse (5′-ACTATGTTAGATTT GGGTCTGGAG-3′) primers. Amplifications were performed with a 5-min denaturation step at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 64°C for 1 min, and extension at 72°C for 1 min. Samples were run on a 0.8% agarose gel. The gels were treated for 2× 30 min in 0.4 M NaOH, 0.6 M NaCl, followed by 2× 30 min in 0.25 M Tris-HCl, pH 7.5, 1.5 M NaCl before blotting onto a Genescreen membrane (NEN Life Science Products, Boston, MA). A 0.9-kb DNA fragment containing the entire human CD20 coding sequence was labeled with ^{32}P using the Megaprime DNA Labeling kit (Amersham Biosciences, Little Chalfont, U.K.) and used for hybridization following standard procedures. To verify that all DNA samples could be amplified by PCR, a normal murine endogenous gene (*ptx3*) was also amplified as a control using specific primers, as described (5′-AGCAATGCACCTCCCTGCGAT-3′; 5′-TCCTCGGTGGGATGAAGTCCA-3′) (22). Briefly, 250 ng DNA was used and amplified for 23 cycles. PCR products were run in a 2% agarose gel.

ADCC and NK assays in vitro

ADCC were performed by standard ^{51}Cr release. Mouse NK effector cells were obtained essentially as described (23). Briefly, murine spleens were collected from age-matched C57BL/6 wild-type or C1qa$^{-/-}$ animals by mechanical disruption. Cells were washed and cultured for 48 h in RPMI 1640 medium containing 10% FCS and 1000 U/ml human rIL-2 (Chiron, Siena, Italy). Cells were then collected, washed, and counted. Target cells were labeled with 30 μCi ^{51}Cr sodium chromate, washed, and incubated for 30 min at 4°C in presence or absence of 10 μg/ml rituximab or 1F5 Ab. A total of 10^4 target cells was incubated in triplicates in U-bottom 96-well plate with increasing amounts of effector cells for 5 h at 37°C or with 0.2% SDS, in 200 μl. A total of 100 μl of supernatant was collected, and released ^{51}Cr was counted in a gamma counter (Wallac Wizard 3; Perkin-Elmer, Shelton, CT). Percentage of lysis was calculated as 100 × (sample release − spontaneous release)/(total release − spontaneous release).

For evaluation of NK activity, the ^{51}Cr-labeled YAC-1 cells were used as target. Mice were either untreated or treated with 100 μg poly(I:C) i.p. (Amersham) 1 day before spleens were collected. Lysis of target cells by spleen effector cells was measured as for ADCC.

Proliferation and viability assays

EL4-CD20$^+$ cells were plated at 10^4 cells/well in 96-well plates in presence or absence of 10 μg/ml rituximab. After 48 h of culture, 0.5 μCi [^{3}H]thymidine (proliferation) or 1/10 vol alamar blue solution (viability) (Biosource, Camarillo, CA) was added in each well, as described (7). Viability assays included a set of wells in which 0.25% Triton X-100 was added 30 min before the alamar blue, to set the background fluorescence (100% dead cells), according to the manufacturer's instructions. Incubation was carried on for an additional 16 h, and plates were either harvested (thymidine) or read in a fluorometer (Cytofluor 2300; Millipore, Bedford, MA), with excitation at 530 nm and emission at 590 nm.

C lysis in vitro

Target cells were labeled with ^{51}Cr sodium chromate and washed twice. Cells were plated at 2×10^4 cells/well in 96-well plates in presence or absence of 10 μg/ml rituximab or 1F5 and freshly drawn mouse (30%) or rat (20%) serum. Incubation was conducted for 5 or 1 h at 37°C, respectively, and an aliquot of supernatant was counted in a gamma counter.

Results

Rituximab or 1F5 Abs eradicate metastatic EL4-CD20$^+$ tumors in vivo

To determine the role of C and ADCC in the therapeutic activity of rituximab in vivo, we have set up a new model of murine lymphoma that stably expresses the human CD20 molecule. For this purpose, we infected the EL4 murine T lymphoma cell line with a retrovirus carrying the human CD20 cDNA coding sequence. The retrovector carried the Moloney murine leukemia virus LTR, which also served as promoter for the transgene (17). Infected CD20-positive cells were sorted by FACS, and the selected cells were shown to stably express high levels of CD20 on 99% of the cells even after several months in continuous culture (Fig. 1*A*). EL4-CD20$^+$ cells express 124,000 CD20 molecules/cell, as determined using calibrated beads, which is comparable to the levels of CD20 observed in primary human B-NHL (range 71,000–170,000 in six cases) or in human lymphoma cell lines (DHL4 and BJAB: 209,000 and 170,000 molecules/cell, respectively) (data not shown).

Injection of 8×10^3 EL4-CD20$^+$ cells in the tail vein of C57BL/6 syngeneic animals produced tumors leading to animal death in 100% of animals within 30–40 days after tumor inoculation (Fig. 1*B*). At necropsy, all animals showed a massive hepatosplenomegaly with evident liver metastases. We did not consistently observe macroscopic lesions in other organs examined, including kidney, bowel, lung, and heart. Nor did we observe macroscopic involvement of the lymph nodes (data not shown). This pattern and time scale of tumor growth were indistinguishable from that obtained with the EL4 wild-type cells (data not shown) and are consistent with previous description of this tumor after i.v. injection (24, 25).

To test rituximab activity and compare it with that of the murine IgG2a anti-CD20 Ab 1F5, we injected groups of animals with 150 μg rituximab i.p., or with an equivalent dose of 1F5 1 day after tumor inoculation. This dose corresponds to the standard dose of 375 mg/m^2 used in the clinic. As control, five animals received the same dose of an irrelevant IgG1 humanized Ab (the anti-human IL-2R β-chain daclizumab), whereas the rest of the animals (n = 12) received saline only after EL4-CD20$^+$ inoculation. As shown in Fig. 1*B*, a single injection of rituximab or 1F5 led to survival of all treated mice, whereas daclizumab had no significant effect. In

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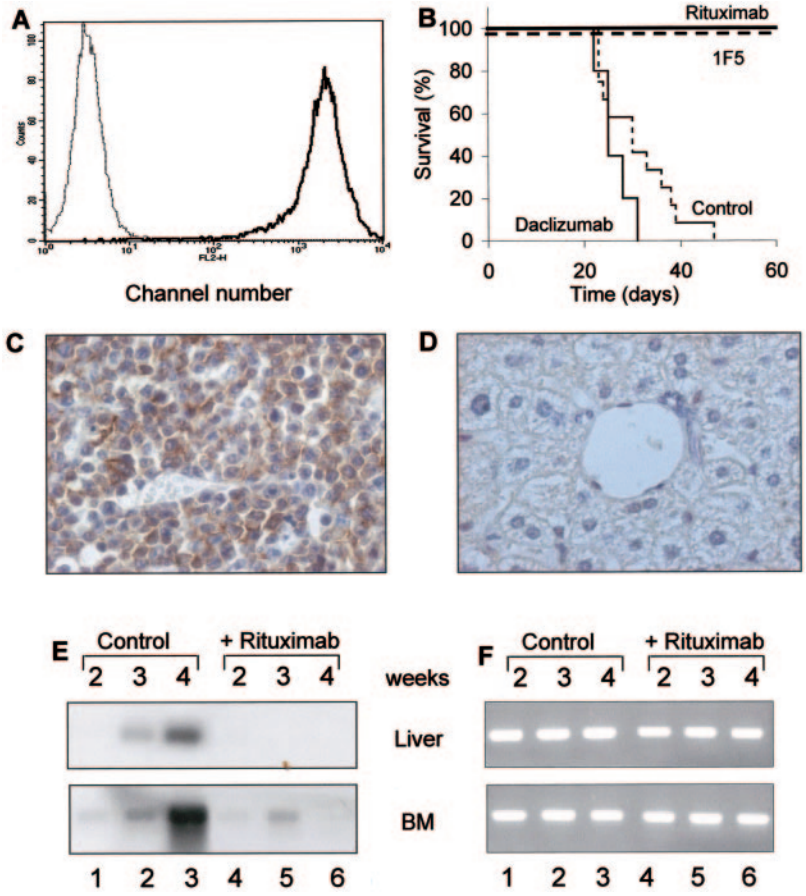

FIGURE 1. Rituximab or 1F5 protects animals from EL4-CD20$^+$-induced death. *A*, Immunofluorescence of EL4-CD20$^+$ cells stained with CD20-PE (thick line) and control Ab (thin line). *B*, Survival curves of C57BL/6 injected i.v. with EL4-CD20$^+$ cells followed by treatment on day 1 with saline (controls, thin dashed line; $n = 12$), rituximab (thick line; $n = 12$), 1F5 (thick dashed line; $n = 6$), or the irrelevant Ab daclizumab (thin line; $n = 5$). The results shown are representative of at least five independent experiments. *C* and *D*, CD20 immunohistochemistry staining of liver sections of a tumor-injected untreated mouse (*C*) and of a rituximab-treated mouse (*D*). Original magnification ×400 in both cases. *E*, Southern blot analysis of PCR-amplified genomic DNA extracted from liver and bone marrow of mice obtained 2–4 wk after tumor inoculation. The 691-bp human-specific band is shown. *F*, Control amplification of an endogenous gene (*ptx3*) with specific oligonucleotides (22), demonstrating integrity and amplificability of all DNA samples shown in *E*. The 124-bp ethidium bromide-stained band is shown.

these experiments, we sacrificed the rituximab-treated animals 120 days after tumor inoculation and found that all animals were still tumor free at this time. Six rituximab-treated animals were kept longer and were still free of tumor 5 mo after tumor cell inoculation. We reproducibly obtained the same results in a series of at least five consecutive experiments.

We performed immunoperoxidase staining of liver and spleen sections excised 4 wk after EL4-CD20$^+$ inoculation and using an Ab specific for human CD20. Livers showed a diffuse metastatic infiltration of CD20$^+$ lymphoma cells (Fig. 1*C*). Similarly, spleen sections showed massive infiltration of CD20-positive lymphoma cells (data not shown). On the contrary, the livers and spleens of animals inoculated with tumor cells followed by rituximab treatment did not show evidence of tumor cell infiltration and retained their normal tissue architecture (Fig. 1*D* and data not shown).

We also followed tumor growth by PCR analysis using primers specific for the human CD20 cDNA. We purified DNA from different organs excised from pairs of animals sacrificed at weekly intervals after tumor cell inoculation. EL4-CD20$^+$ cells could already be detected by PCR at week 2 after tumor cell inoculation in bone marrow and spleen and at week 3 in liver (Fig. 1*E*, *lanes 1* and *2*, and data not shown). The presence of EL4-CD20$^+$ cells was very evident in all organs at week 4 (Fig. 1*E*, *lane 3*). In contrast, in animals inoculated with EL4-CD20$^+$ cells and treated with rituximab, the presence of EL4-CD20$^+$ cells could be detected at weeks 2–3 in bone marrow and spleen, but was undetectable in all organs at week 4 (Fig. 1*E*, *lanes 4–6*, and data not shown). These data show that rituximab has a relatively slow effect on tumor cell growth because CD20$^+$ cells could still be detected at weeks 2–3 in treated animals in spleen and bone marrow. As a control, all DNA samples were also amplified with primers specific for an endogenous mouse gene (*ptx3*). The data show that the control gene could be amplified from all DNA samples, demonstrating the integrity of all DNA preparations used (Fig. 1*F*).

We also determined whether rituximab maintained therapeutic activity even if given at later time points. The same experiment was therefore performed, inoculating 8×10^3 EL4-CD20$^+$ cells i.v. on day 0 and 250 μg rituximab i.p. on day 1, 2, or 3. As shown in Fig. 2, although retarding rituximab administration diminished slightly the therapeutic effect, still 80 and 60% of the animals were cured when the Ab was given at day 2 or 3, respectively.

These data show that a single injection of rituximab or murine Ab 1F5, inoculated by a different route and up to 3 days after the tumor cells, leads to long-term protection from tumor growth and death in animals inoculated with metastatic syngeneic lymphoma cells.

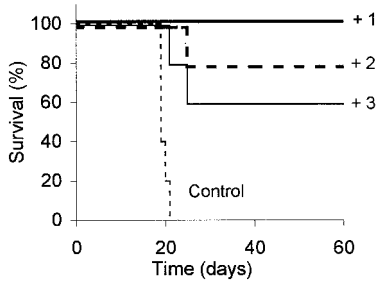

FIGURE 2. Rituximab shows therapeutic activity even when given 3 days after the tumor. Survival curve of groups of animals inoculated i.v. with 8×10^3 EL4-CD20$^+$ cells and treated with saline (thin dashed line) or rituximab on days +1 (thick continuous line), +2 (thick dashed line), or +3 (thin continuous line).

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Rituximab does not affect EL4-CD20$^+$ cell growth or viability in vitro

Rituximab has been shown previously to directly inhibit the proliferation or induce apoptosis of some human leukemic B cells in vitro (26). Thus, the therapeutic activity of rituximab in vivo in our syngeneic mouse model may have been due to a direct effect of rituximab on the growth of EL4-CD20$^+$ cells. This point was therefore investigated. EL4-CD20$^+$ cells were cultured in presence or absence of rituximab for 64 h. Proliferation was measured by a standard thymidine uptake assay and viability using the alamar blue dye (7, 27). The data shown in Fig. 3 demonstrate that rituximab did not affect either proliferation or viability in vitro. The same results were obtained after 4 days of treatment with rituximab (data not shown).

These results demonstrate that rituximab has no direct effect on the growth or viability of EL4-CD20$^+$ cells in vitro.

Depletion of NK, PMN, or T cells does not affect rituximab therapeutic activity

Rituximab is a chimeric Ab carrying the human IgG1 Fc portion. To verify that rituximab was able to activate ADCC with murine effector cells, we performed ADCC assays using the EL4-CD20$^+$ cells as target and murine splenic cells as effectors. As positive control, the assay was also performed with the murine 1F5 Ab. Murine splenic cells were able to lyse 75% of EL4-CD20$^+$ targets after 5-h incubation in presence of either rituximab or 1F5, with 15–20% lysis above background in absence of Ab (Fig. 4*A*). These data demonstrate that rituximab and 1F5 can mediate ADCC of the EL4-CD20$^+$ target cells by C57BL/6 splenic cells.

We have next determined whether NK cells are involved in mediating the therapeutic activity of rituximab in vivo. We first verified that the anti-NK Ab TM-β1 (anti-murine IL-2R β-chain) did not recognize the EL4-CD20$^+$ lymphoma cells (data not shown). Groups of C57BL/6 mice (n = 6 per group) were then depleted of NK cells by repeated injection of TM-β1, as described previously (19), starting 2 days before tumor cell inoculation. We then inoculated the standard dose of EL4-CD20$^+$ cells in both treated and control mice, followed or not by the standard dose of rituximab 1 day after. Both control and anti-NK-treated animals died within 31 days following tumor cell inoculation, as expected. Rituximab was able to cure 100% of the animals in both the control and anti-NK-treated groups (Fig. 5*A*). Also, in this case, we sacrificed all rituximab-treated animals at 120 days and over and found no evidence of tumor growth in both groups. These data show that NK cells are not required for rituximab activity in vivo. That Ab treatment had depleted NK cells was shown in separate groups of animals by complete inhibition of NK activity against the YAC1 target, 2 days after treatment (data not shown), in agreement with published results (19).

PMN may contribute to ADCC by activation through their FcγRIII receptor (28, 29), because mouse PMN do not express FcγRI (30). We therefore investigated their possible role in the response to rituximab by depletion with the PMN-specific anti-GR1 mAb RB6-8C5, as described previously (20). Ab treatment led after 2 days to a 91% (±3%) decrease of peripheral blood PMN compared with untreated animals (data not shown), in agreement with previous results (20). Also, in this case, PMN depletion

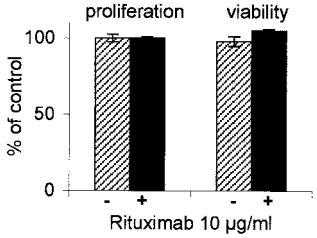

FIGURE 3. Rituximab does not affect EL4-CD20$^+$ in vitro growth. EL4-CD20$^+$ cells (5×10^4/ml) were plated in absence (dashed bars) or presence (filled bars) of 10 μg/ml rituximab. Cell proliferation ([^{3}H]thymidine uptake) and the number of live cells (alamar blue assay) were evaluated after 64 h. The results are expressed as percentage of the control cells cultured in absence of rituximab. The results are representative of two independent experiments.

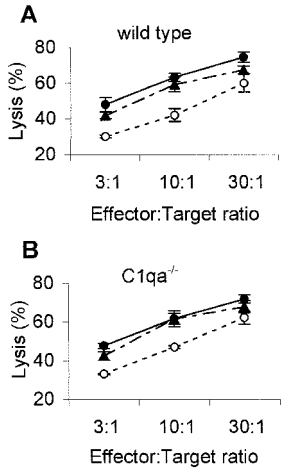

FIGURE 4. Rituximab mediates ADCC in vitro with murine splenic effector cells. A total of 10^4 ^{51}chromium-labeled EL4-CD20$^+$ target cells was incubated in the presence of rituximab (●), 1F5 (▲), or absence of Ab (○). After washing, they were incubated for 5 h with murine splenic effector cells at the indicated ratios, and release of label was quantified. Effector cells were obtained either from C57BL/6 wild-type (*A*) or C1qa^{-}/$^{-}$ mice (*B*). The results are representative of two independent experiments.

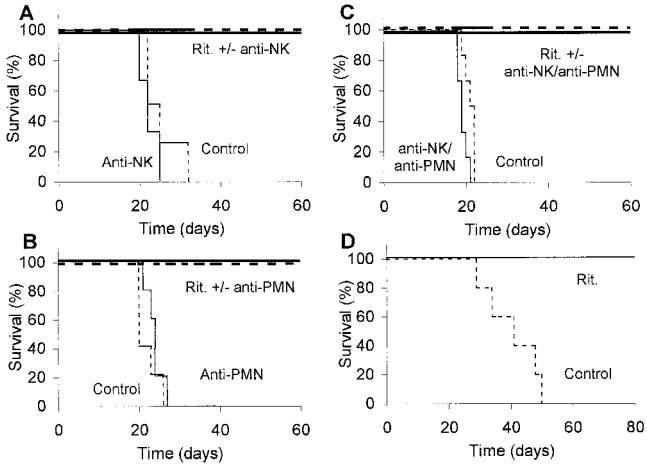

FIGURE 5. In vivo depletion of NK, PMN, or T cells does not affect rituximab efficacy. Mice (n = 5 per group) were repeatedly injected i.p. with the anti-NK Ab TM-β1 (*A*), the anti-PMN Ab RB-8C5 (*B*), both Abs (*C*) (continuous lines), or control saline (dashed lines), starting 2 days before EL4-CD20$^+$ tumor cells injection. All mice were inoculated i.v. with EL4-CD20$^+$ cells and then either treated with rituximab (thick lines) or with saline (thin lines) the day after. *D*, Irradiated nude mice (five/group) were inoculated i.v. with EL4-CD20$^+$ cells, followed by 150 μg rituximab (continuous line) or saline (dashed line) 1 day after. The results are representative of two independent experiments.

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did not affect tumor growth in animals inoculated with EL4-CD20$^+$ cells, nor did it affect the therapeutic efficacy of rituximab, because all anti-PMN-treated and control animals were cured by rituximab up to 3 mo after tumor cell inoculation (Fig. 5*B*).

We also tested the effect of depletion of both NK and PMN cells simultaneously, using the same protocols as above. As shown in Fig. 5*C*, rituximab still efficaciously cured animals injected with the standard dose of EL4-CD20$^+$ cells, even after in vivo depletion of both NK and PMN cells.

Because T cells have been reported to play a role in the protection of animals from EL4 tumor under some experimental conditions (31), we have tested the role of T cells in rituximab activity, using irradiated nude athymic mice. Injection of 8×10^3 EL4-CD20$^+$ cells i.v. in these animals led to tumor growth, resulting in death within 30–50 days (Fig. 5*D*). The administration of the standard 150 μg dose of rituximab 1 day after tumor led to survival of 100% of the animals for at least 100 days (Fig. 5*D*).

Altogether, these data demonstrate that NK cells, PMN, and T lymphocytes are dispensable for rituximab therapeutic activity in vivo against EL4-CD20$^+$ cells.

C1q is required for the therapeutic activity of rituximab

Rituximab activates CDC efficiently in vitro using human lymphoma cells and human serum (7, 8). Before investigating the role of C in vivo, we first verified that rituximab was able to activate mouse C in vitro. Addition of 10 μg/ml rituximab or 1F5 led to lysis of 21% ($\pm$4%) and 18% ($\pm$3%) of EL4-CD20$^+$ cells after 5 h in presence of 30% freshly drawn mouse serum (data not shown), respectively. Serum alone had no effect. Because mouse serum is known to be poorly lytic in vitro (32), we also performed CDC in presence of 20% rat serum. In this case, both rituximab and 1F5 led to over 95% lysis of EL4-CD20$^+$ cells (data not shown). These data demonstrate that rituximab can activate rodent C in vitro with the same efficiency as the murine IgG2a Ab 1F5.

To determine the role of C in vivo, we used syngeneic knockout animals lacking completely C1q, the first component of the classical pathway of C activation (C1qa$^{-/-}$) (21). Inoculation of the same dose of EL4-CD20$^+$ cells in 15 animals led to tumor growth and animal death indistinguishable from that observed in the wild-type animals (Fig. 6*A*). In a parallel group of 15 animals, however, injection of the standard dose of rituximab did not alter in any way the survival curve, suggesting that tumor growth was unaffected by Ab treatment (Fig. 6*A*). Similar experiments were performed with the 1F5 Ab. Again, 1F5 was unable to eradicate tumors in C1q$^{-/-}$ animals (Fig. 6*B*). In experiments with C1qa$^{-/-}$ animals, we conducted parallel control experiments with wild-type animals that were reproducibly cured by rituximab (Fig. 1*B*, and data not shown). At necropsy, both rituximab-treated and control C1qa$^{-/-}$ animals showed similar splenomegaly and liver metastases. As expected, the presence of extensive infiltration of EL4-CD20$^+$ cells could be demonstrated in the liver by immunohistochemistry in both control (Fig. 6*C*) and rituximab-treated animals (Fig. 6*D*). Finally, PCR analysis clearly demonstrated the presence of tumor cells starting at week 2 in bone marrow and spleen and at weeks 3–4 in liver in both rituximab-treated and control animals (Fig. 6*E*). As before, amplification of the control endogenous gene *ptx3* demonstrated the integrity of all DNA samples analyzed (Fig. 6*F*).

To verify that C1qa$^{-/-}$ animals are capable of ADCC, we conducted cytotoxicity assays in vitro using spleen cells from C1qa$^{-/-}$ animals as effectors and the EL4-CD20$^+$ cells as targets.

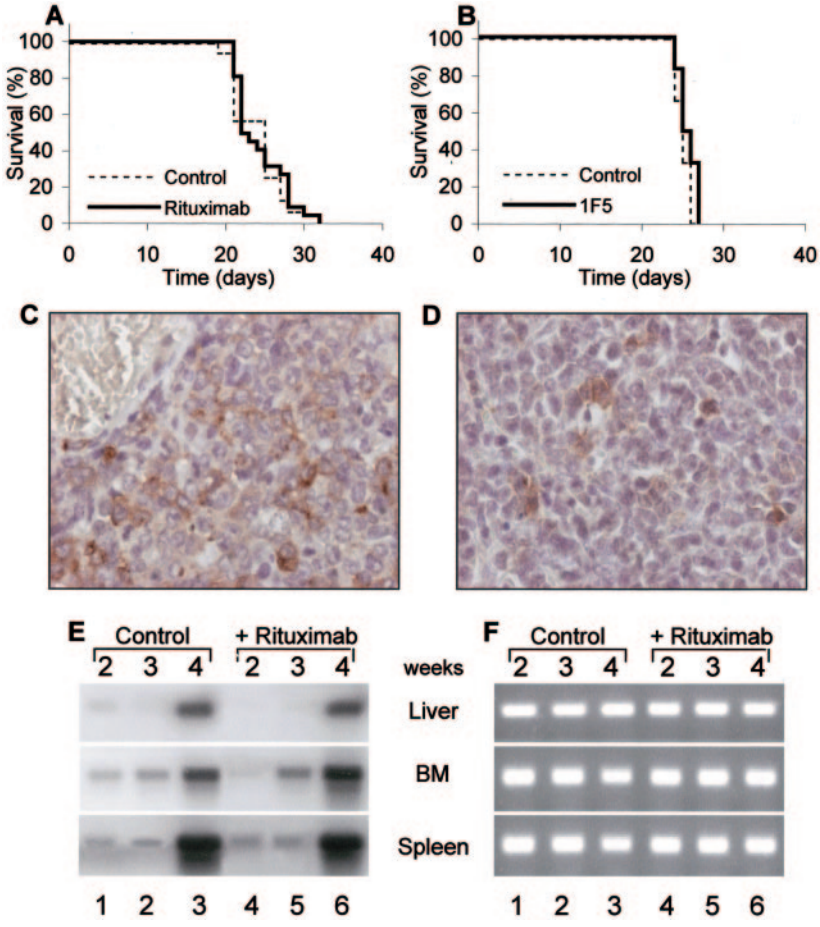

FIGURE 6. C activity is required for rituximab therapeutic effect. *A* and *B*, Survival curves of C1qa$^{-/-}$ animals (C57BL/6 background) injected i.v. with EL4-CD20$^+$ cells and either treated with rituximab (solid line, *A*; $n = 15$ per group), 1F5 (solid line, *B*; $n = 6$ per group), or control saline (dashed lines, *A* and *B*). *C* and *D*, CD20 immunohistochemistry staining of liver sections of control (*C*) or rituximab-treated C1qa$^{-/-}$ mice (*D*). Original magnification $\times$400. *E*, Southern blot analysis of PCR-amplified DNA extracted from liver, bone marrow, and spleen of C1qa$^{-/-}$ mice obtained 2, 3, or 4 wk after tumor inoculation. The 691-bp human-specific band is shown. *F*, Control amplification of an endogenous gene (*ptx3*) with specific oligonucleotides (22), demonstrating integrity and amplificability of all DNA samples shown in *E*. The 124-bp ethidium bromide-stained band is shown.

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C1qa$^{-/-}$ splenic cells lysed EL4-CD20$^+$ cells to 73% in presence of rituximab or 1F5 and up to 15% above background without Ab (Fig. 4*B*), similar to what was obtained with wild-type spleen cells (Fig. 4*A*). Thus, C1qa$^{-/-}$-derived spleen cells are not significantly different from those of wild-type animals in their ADCC activity.

Altogether, these data clearly demonstrate that rituximab or 1F5 is unable to eradicate tumor cells or check tumor growth in vivo in the absence of C1q.

Discussion

Our data demonstrate unequivocally, using a new nonimmunodeficient murine model, that C1q, and therefore classical C activation, is a fundamental element for rituximab therapeutic activity in vivo, whereas NK, PMN, or T cells are dispensable. Because the same results were obtained with the murine IgG2a anti-CD20 Ab 1F5, which itself activates C efficiently (33) (data not shown), the results obtained were not due to the use of a humanized Ab in mice. These data are consistent with previous findings in vitro that rituximab lyses human lymphoma cells more efficiently through CDC than through ADCC in vitro (8, 33–35). Furthermore, an IgG4 version of the molecule that does not activate CDC nor ADCC does not deplete peripheral blood B cells in nonhuman primates (36), and C has been shown to be rapidly activated in vivo after rituximab infusion (10, 11). Additional indirect evidence for a role of C in vivo comes from the apparent correlation between CDC in vitro and clinical response in B-CLL vs follicular lymphoma cells (7, 9, 37, 38).

In our model, we can exclude a role for apoptosis because we could not detect any induction of apoptosis or inhibition of EL4-CD20$^+$ cell growth in vitro in response to rituximab. Indeed, our results on the role of C in the therapeutic activity of both rituximab and 1F5 are in agreement with those obtained by Glennie and colleagues (33) using a xenograft model of lymphoma and C depletion by cobra venom factor (M. Cragg and M. Glennie, personal communication). Thus, a role for C in vivo does not seem to be restricted to the EL4-CD20$^+$ model.

In our model, we injected EL4-CD20$^+$ cells i.v. to allow dissemination of the tumor cells into hemopoietic organs (spleen and bone marrow), thus more closely mimicking hematological neoplasias. Furthermore, the therapeutic mAbs were given by a different route (i.p.) to avoid rapid C-mediated lysis of tumor cells in the circulation before their homing into the different organs. Interestingly, we could detect by PCR EL4-CD20$^+$ cells in rituximab-treated animals up to weeks 2–3 in some organs (spleen and bone marrow). This finding suggests that rituximab did not eliminate all tumor cells immediately, but that tumor cell eradication was relatively slow, more similar to that observed in B-NHL patients treated with this Ab. In agreement with a gradual effect of rituximab in vivo, we could show that rituximab still showed strong, although not maximal, therapeutic activity even when given up to 3 days after the tumor. Thus, the therapeutic activity of the mAb was not due simply to immediate elimination of few tumor cells.

In contrast to a requirement for C, a study by Clynes et al. (13) suggested an important role of ADCC in the mechanism of action of rituximab. In their model, rituximab loses full therapeutic activity against a human B lymphoma xenograft in the absence of the FcγR γ-chain common to both FcγRI and FcγRIII. A possible explanation for these divergent results is the different model used (an s.c. xenograft in nude mice). Alternatively, C activation may synergize with FcγR-mediated immune mechanisms. Classical pathway activation leads to target cell opsonization through deposition of C3 and C4 fragments on the cell surface, leading to augmented phagocytosis (39). iC3b deposition also increases ADCC by NK, PMN, and macrophages that express the CR3 receptor in addition to FcγR (40, 41). Soluble C fragments, in particular C3a and C5a, stimulate strongly the inflammatory response and are chemotactic for neutrophils and other inflammatory cells. C5a also increases FγRIII and decreases FcγRII expression on macrophages (42). Finally, completion of the C cascade leads to cell lysis through formation of the membrane attack complex containing the C fragments C5b-9 (40). Thus, activation of the C pathway may activate several immune-mediated mechanisms in addition to inducing direct cell lysis. Indeed, recently the CR3 receptor has been shown to participate in the control of melanoma growth by the IgG2a therapeutic mAb TA99 (41), suggesting an interaction between C and ADCC in this model. The data presented in this work showing that NK, PMN, as well as T cells are dispensable for the therapeutic activity of rituximab, however, point to a major role of C-mediated lysis and/or removal by scavenger cells for tumor eradication, at least in our EL4-CD20$^+$ tumor model.

Our results on a role of C in the therapeutic activity of rituximab has homologies to the recent findings obtained with a model of vitiligo, an Ab-mediated antoimmune disease. In this model, Ab-mediated autoimmunity depends on both C and FcγR (43).

To conclude, this is the first demonstration in vivo of the fundamental role of C activation in the therapeutic activity of rituximab. These conclusions have important implications in the design of new improved version of rituximab, such as point mutants with increased C-activating function (44) or bispecific anti-CD20/CD55 molecules, as well as of other unconjugated IgG1 anti-cancer Abs (1). The murine model presented should prove useful in testing such strategies in vivo.

References

1. Carter, P. 2001. Improving the efficacy of antibody-based cancer therapies. *Nat. Rev. Cancer 1:118.*
2. White, C. A., R. L. Weaver, and A. J. Grillo-Lopez. 2001. Antibody-targeted immunotherapy for treatment of malignancy. *Annu. Rev. Med. 52:125.*
3. Reff, M. E., C. Carner, K. S. Chambers, P. C. Chinn, J. E. Leonard, R. Raab, R. A. Newman, N. Hanna, and D. R. Anderson. 1994. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood 83:435.*
4. McLaughlin, P., C. A. White, A. J. Grillo-Lopez, and D. G. Maloney. 1998. Clinical status and optimal use of rituximab for B cell lymphomas. *Oncology 12:1763.*
5. Coiffier, B., E. Lepage, J. Briere, R. Herbrecht, H. Tilly, R. Bouabdallah, P. Morel, E. Van den BNeste, G. Salles, P. Gaulard, et al. 2002. CHOP chemotherapy plus rituximab compared to chop alone in elderly patients with diffuse large B cell lymphoma. *N. Engl. J. Med. 346:235.*
6. Foran, J. M., A. Z. Rohatiner, D. Cunningham, R. A. Popescu, P. Solal-Celigny, M. Ghielmini, B. Coiffier, P. W. Johnson, C. Gisselbrecht, F. Reyes, et al. 2000. European phase II study of rituximab (chimeric anti-CD20 monoclonal antibody) for patients with newly diagnosed mantle-cell lymphoma and previously treated mantle-cell lymphoma, immunocytoma, and small B-cell lymphocytic lymphoma. *J. Clin. Oncol. 18:317.*
7. Golay, J., M. Lazzari, V. Facchinetti, S. Bernasconi, G. Borleri, T. Barbui, A. Rambaldi, and M. Introna. 2001. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood 98:3383.*
8. Golay, J., L. Zaffaroni, T. Vaccari, M. Lazzari, G. M. Borleri, S. Bernasconi, F. Tedesco, A. Rambaldi, and M. Introna. 2000. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. *Blood 95:3900.*
9. Belosillo, B., N. Villamor, A. Lopez-Guillermo, S. Marcé, J. Esteve, E. Campo, D. Colomer, and E. Montserrat. 2001. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated in vitro by caspase-independent mechanism involving the generation of reactive oxygen species. *Blood 98:2771.*
10. Van der Kolk, L. E., A. J. Grillo-Lopez, J. W. Baars, C. E. Hack, and M. H. van Oers. 2001. Complement activation plays a key role in the side-effects of rituximab treatment. *Br. J. Haematol. 115:807.*
11. Kennedy, A. D., M. D. Solga, T. A. Schuman, A. W. Chi, M. A. Lindorfer, W. M. Sutherland, P. L. Foley, and R. P. Taylor. 2003. An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20$^+$ cells by rituximab. *Blood 101:1071.*
12. Cartron, G., L. Dacheux, G. Salles, P. Solal-Celigny, P. Bardos, P. Colombat, and H. Watier. 2002. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγIIIa gene. *Blood 99:754.*
13. Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med. 6:443.*

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14. Shan, D., J. A. Ledbetter, and O. W. Press. 1998. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. *Blood 91:1644.*
15. Pedersen, I. M., A. M. Buhl, P. Klausen, C. H. Geisler, and J. Jurlander. 2002. The chimeric anti-CD20 antibody rituximab induces apoptosis in B-cell chronic lymphocytic leukemia cells through a p38 mitogen activated protein-kinase-dependent mechanism. *Blood 99:1314.*
16. Byrd, J. C., S. Kitada, I. W. Flinn, J. L. Aron, M. Pearson, D. Lucas, and J. C. Reed. 2002. The mechanism of tumor cell clearance by rituximab in vivo in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. *Blood 99:1038.*
17. Introna, M., A. Barbui, F. Bambacioni, C. Casati, G. Gaipa, G. M. Borleri, S. Bernasconi, T. Barbui, J. Golay, A. Biondi, and A. Rambaldi. 2000. Genetic modification of human T cells with CD20: a strategy to purify and lyse transduced cells with anti-CD20 antibodies. *Hum. Gene Ther. 11:611.*
18. Golay, J. T., E. A. Clark, and P. C. Beverley. 1985. The CD20 (Bp35) antigen is involved in activation of B cells from the G_0 to the G_1 phase of the cell cycle. *J. Immunol. 135:3795.*
19. Tanaka, T., F. Kitamura, Y. Nagasaka, K. Kuida, H. Suwa, and M. Miyasaka. 1993. Selective long-term elimination of natural killer cells in vivo by an anti-interleukin 2 receptor β chain monoclonal antibody in mice. *J. Exp. Med. 178: 1103.*
20. Pruijt, J. F. M., P. Verzaal, R. van Os, E. F. M. de Kuijf, M. L. J. van Schie, A. Mantovani, A. Vecchi, I. J. D. Lindley, R. Willemze, S. Starckx, et al. 2002. Neutrophils are indispensable for hematopoietic stem cell mobilization induced by interleukin-8 in mice. *Proc. Natl. Acad. Sci. USA 99:6228.*
21. Botto, M., M. C. Dell'Agnola, A. E. Bygrave, E. M. Thompson, H. T. Cook, F. Petry, M. Loos, P. P. Pandolfi, and M. J. Walport. 1998. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat. Genet. 19:56.*
22. Garlanda, C., E. Hirsch, S. Bozza, A. Salustri, M. De Acetis, R. Nota, A. Maccagno, F. Riva, B. Bottazzi, G. Peri, et al. 2002. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature 420:182.*
23. Arase, H., T. Suenaga, N. Arase, Y. Kimura, K. Ito, R. Shiina, H. Ohno, and T. Saito. 2001. Negative regulation of expression and function of FcγRIII by CD3ζ in murine NK cells. *J. Immunol. 166:21.*
24. Ding, L., M. Sunamura, T. Kodama, J. Yamauchi, D. G. Duda, H. Shimamura, H. Shibuya, K. Takeda, and S. Matsuma. 2001. In vivo evaluation of the early events associated with liver metastasis of circulating cancer cells. *Br. J. Cancer 85:431.*
25. Seki, S., W. Hashimoto, K. Ogasawara, M. Satoh, H. Watanabe, Y. Habu, H. Hiraide, and K. Takeda. 1997. Antimetastatic effect of $NK1^+$ T cells on experimental haematogenous tumour metastases in the liver and lungs of mice. *Immunology 92:561.*
26. Rose, A. L., B. E. Smith, and D. G. Maloney. 2002. Glucocorticoids and rituximab in vitro: synergistic direct anti-proliferative and apoptotic effects. *Blood 100:1765.*
27. Gazzano-Santoro, H., P. Ralph, T. C. Ryskamp, A. B. Chen, and V. R. Mukku. 1997. A non-radioactive complement-dependent cytotoxicity assay for anti-CD20 monoclonal antibody. *J. Immunol. Methods 202:163.*
28. Van der Kolk, L. E., M. de Haas, A. J. Grillo-Lopez, J. W. Baars, and M. H. van Oers. 2002. Analysis of CD20-dependent cellular cytotoxicity by G-CSF-stimulated neutrophils. *Leukemia 16:693.*
29. Valerius, T., R. Repp, T. P. de Wit, S. Berthold, E. Platzer, J. R. Kalden, M. Gramaski, and J. G. van de Winkel. 1993. Involvement of the high affinity receptor for IgG (FcγRI; CD64) in enhanced tumor cell cytotoxicity of neutrophils during granulocyte colony-stimulating factor therapy. *Blood 82:931.*
30. Ioan-Facsinay, A., S. J. de Kimpe, S. M. Hellwig, P. L. van Lent, F. M. Hofhuis, H. H. van Ojik, C. Sedlik, S. A. da Silveira, J. Gerber, Y. F. de Jong, et al. 2002. FcγRI (CD64.) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. *Immunity 16:391.*
31. Runyon, K., K. Lee, K. Zuberek, M. Collins, J. P. Leonard, and K. Dunussi-Joannopoulos. 2001. The combination of chemotherapy and systemic immunotherapy with soluble B7-immunoglobulin G leads to cure of murine leukemia and lymphoma and demonstration of tumor-specific memory responses. *Blood 97:2420.*
32. Miwa, T., L. Zhou, B. Hilliard, H. Molina, and W. C. Song. 2002. Crry, but not CD59 and DAF, is indispensable for murine erythrocyte protection in vivo from spontaneous complement attack. *Blood 99:3707.*
33. Cragg, M. S., S. M. Morgan, H. T. Chan, B. P. Morgan, A. V. Filatov, P. W. Johnson, R. R. French, and M. J. Glennie. 2003. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. *Blood 101: 1045.*
34. Di Gaetano, N., Y. M. Xiao, E. Erba, R. Bassan, A. Rambaldi, J. Golay, and M. Introna. 2001. Synergism between fludarabine and rituximab revealed in a follicular lymphoma cell line resistant to the cytotoxic activity of either drug alone. *Br. J. Haematol. 114:1.*
35. Weiner, G. J. 2003. Rituximab: complementary mechanisms of action. *Blood 101:788.*
36. Anderson, D. R., A. Grillo-Lopez, C. Varns, K. S. Chambers, and N. Hanna. 1997. Targeted anti-cancer therapy using rituximab, a chimaeric anti-CD20 antibody (IDEC-C2B8) in the treatment of non-Hodgkin's B-cell lymphoma. *Biochem. Soc. Trans. 25:705.*
37. Keating, M. J., S. O'Brien, and M. Albitar. 2002. Emerging information on the use of rituximab in chronic lymphocytic leukemia. *Semin. Oncol. 29:70.*
38. Manches, O., G. Lui, L. Chaperot, R. Gressin, J. P. Molens, M. C. Jacob, J. J. Sotto, D. Leroux, J. C. Bensa, and J. Plumas. 2003. In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood 101:949.*
39. Barrington, R., M. J. Zhang, P. Fischer, and M. C. Carroll. 2001. The role of complement in inflammation and adaptive immunity. *Immunol. Rev. 180:5.*
40. Gorter, A., and S. Meri. 1999. Immune evasion of tumor cells using membrane-bound complement regulatory proteins. *Immunol. Today 20:576.*
41. Van Spriel, A. M., H. H. van Ojik, A. Bakker, M. J. H. Jansen, and J. J. G. van de Winkel. 2003. Mac-1 (CD11b/CD18) is crucial for effective Fc receptor-mediated immunity to melanoma. *Blood 101:253.*
42. Shushakova, N., J. Skokowa, J. Schulman, U. Baumann, J. Zwirner, R. E. Schmidt, and J. E. Gessner. 2002. C5a anaphylatoxin is a major regulator of activating versus inhibitory FcγRs in immune complex-induced lung disease. *J. Clin. Invest. 110:1823.*
43. Trcka, J., Y. Moroi, R. A. Clynes, S. M. Glodberg, A. Bergtold, M.-A. Perales, M. Ma, C. R. Ferrone, M. C. Carroll, J. V. Ravetch, and A. N. Houghton. 2002. Redundant and alternative roles for activating Fc receptors and complement in an antibody-dependent model of autoimmune vitiligo. *Immunity 16:861.*
44. Idusogie, E. E., P. Y. Wong, L. G. Presta, H. Gazzano-Santoro, K. Totpal, M. Ultsch, and M. G. Mulkerrin. 2001. Engineered antibodies with increased activity to recruit complement. *J. Immunol. 166:2571.*

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