



The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes

Josée Golay
Elena Cittera
Nicola Di Gaetano
Massimiliano Manganini
Michela Mosca
Manuela Nebuloni
Nico van Rooijen
Luca Vago
Martino Introna

Background and Objectives. We have set up a murine B lymphoma model stably expressing human CD20 and homing in lymph nodes in immunocompetent mice to study the mechanism of action of rituximab.

Design and Methods. The B lymphoma line 38C13 was stably transduced with the human CD20 cDNA by retroviral infection and injected into syngeneic mice.

Results. The transduced 38C13-CD20⁺ cells stably expressed human CD20 on 100% of cells. Rituximab alone did not inhibit 38C13-CD20⁺ cell growth but relocalized the human CD20 into lipid rafts and induced complement-mediated lysis *in vitro*. Inoculation of 4×10³ 38C13-CD20⁺ intravenously into syngeneic mice led to the development of tumor masses in the spleen, bone marrow and lymph nodes, detectable from day 15 by polymerase chain reaction (PCR) analysis, and with a median survival of 21-24 days. Treatment with 250 µg rituximab i.p. given 1-10 days after tumor inoculation cured 100% of animals, with disappearance of tumor documented by immunohistochemistry and PCR analysis. Depletion of both NK cells and neutrophils did not affect the therapeutic activity of rituximab *in vivo*. Similarly, removal of phagocytic macrophages using clodronate-liposomes did not modify the capacity of rituximab to control tumor growth. In contrast, the protective activity of the antibody was completely abolished after complement depletion with cobra venom factor. Complement was also required when cells were inoculated subcutaneously in nude mice.

Interpretation and Conclusions. These data demonstrate that complement is required for the therapeutic activity of rituximab *in vivo* in a murine model of B-cell lymphoma, independently of its localization in lymph nodes or subcutaneously.

Key words: B lymphoma, rituximab, complement.

Haematologica 2006; 91:176-183

©2006 Ferrata Storti Foundation

From the Laboratory of Cellular and Gene Therapy "G. Lanzani", Division of Haematology, Ospedali Riuniti di Bergamo, Bergamo, Italy (JG, MM, MI); Department of Immunology and Cell Biology, Istituto Ricerche Farmacologiche Mario Negri, Milan, Italy (EC, NDG, MM); Institute of Pathology, Department of Clinical Sciences "L. Sacco", University of Milan, Milan, Italy (MN, LV); Vrije Universiteit, VUMC, Department of Molecular Cell Biology, Faculty of Medicine, Amsterdam, The Netherlands (NvR).

Correspondence:

Josée Golay, Laboratory of Cellular and Gene Therapy "G. Lanzani", c/o Presidio Matteo Rota, via Garibaldi 11-13, Ospedali Riuniti di Bergamo, 24128 Bergamo, Italy.
E-mail: jgolay@ospedaliriuniti.bergamo.it

Rituximab (Mabthera[®], Rituxan[®]) is a chimeric unconjugated monoclonal IgG₁ antibody extensively used for the treatment of low- and high-grade non-Hodgkin's B lymphomas (B-NHL) and with therapeutic activity also in other B-cell neoplasms such as B-cell chronic lymphocytic leukemia, hairy cell leukemia and Waldenström's macroglobulinemia.¹⁻⁶ More recently its B cell depleting activity has found an application for the treatment of several autoimmune diseases, such as rheumatoid arthritis and immune thrombocytopenia.^{7,8} A clear understanding of the mechanism of action of rituximab is a key issue for the development of new more active therapeutic monoclonal antibodies. The activity of rituximab *in vivo* has been proposed by several groups to depend, at least in part, upon its interaction with immune cells, in particular through antibody dependent cellular cytotoxicity (ADCC) and phagocytosis.^{9,10} Indeed the FcγR γ chain, which is required for signaling from the activating FcγRI and FcγRIII receptors present on the surface of macrophages, neutrophils and natural killer (NK) cells, has been shown to be required for the full therapeutic activity of rituximab in a subcutaneous xenograft model of lymphoma.¹¹ More recently a role for both NK cells and neutrophils has also been demonstrated in a disseminated xenograft model in severe combined immunodeficiency (SCID) mice.¹² In man, the role of ADCC is supported by the

reported correlation between FcγRIIIA and IIA polymorphisms and clinical response to rituximab in patients with follicular lymphoma and Waldenström's macroglobulinemia,^{6,13,14} although this finding has not been confirmed in B-cell chronic lymphocytic leukemia.¹⁵ Much disagreement does, however, remain on the role of complement in the therapeutic activity of rituximab. We and others have shown that rituximab lyses lymphoma cells and primary samples very efficiently *in vitro*¹⁶⁻¹⁹ in the presence of complement, suggesting that this mechanism may also be important *in vivo*. Furthermore, complement activation has been shown to take place rapidly *in vivo* following rituximab administration,^{20,21} although this activation has been linked by some authors to the infusion-related effects of the antibody.²¹ We have recently reported on a new CD20⁺ lymphoma model in immunocompetent mice generated by stably introducing the human CD20 cDNA in a murine T lymphoma cell line, EL4. Rituximab could fully cure mice inoculated i.v. with EL4-CD20⁺ cells, and depletion of NK cells, polymorphonuclear cells (PMN) or both, or that of T cells did not affect the therapeutic activity of the antibody. In contrast rituximab was inactive in syngeneic C1q knock-out animals lacking the first component of the complement cascade.²² These data unequivocally showed that complement is required for the therapeutic activity of rituximab, whereas cell-

mediated mechanisms were not, at least in this metastatic T lymphoma model. An important role for complement has also been shown in a xenograft model in SCID mice, in which complement depletion with cobra venom factor (CVF) nearly abolished the therapeutic activity of rituximab.²³ However, more recently, the role of complement has been questioned by Uchida *et al.* who used a completely murine model in which the mechanism of normal B-cell depletion *in vivo* was studied using murine anti-mouse CD20 antibodies.²⁴ B-cell depletion in these mice was efficient even in the absence of complement but did require FcγRs.²⁴ In order to try and resolve this issue and with the idea that fully immunocompetent models may more adequately represent a naturally occurring human lymphoma, we have set up a new syngeneic B lymphoma model that homes into lymph nodes, unlike the previously characterized EL4 lymphoma, which is of T-cell origin and rapidly metastasizes into the liver.²² For this purpose, the murine B lymphoma cell line 38C13 was stably transduced with human CD20 cDNA and injected into syngeneic mice. We then investigated the therapeutic activity of rituximab at different time points in this model as well as the role of complement and immune cells.

Design and Methods

Infection and selection of 38C13-CD20⁺ cells

38C13 murine B lymphoma cells were kindly provided by Prof. J. Haimovich (Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Israel) and were cultured in RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (HyClone, Steril System, Logan, UT, USA), 2 mM glutamine (Life Technologies, Paisley, Scotland), 50 μM β-mercaptoethanol and 100 IU/mL penicillin/streptomycin (hereafter called complete RPMI1640). 38C13 cells were infected with the lentiviral vector pRRLsin.PPTs.EFα.CD20pre containing the human CD20 cDNA, as previously described.²⁵ Briefly 293T cells were grown in Iscove's modified Dulbecco's medium (Life Technologies) supplemented with 10% fetal calf serum and glutamine and transfected with 3.5 μg of envelope plasmid pMD.G, 6.5 μg of packaging construct pCMVdr8.74 and 10 μg of transfer construct pRRLsin.PPTs.EF1α.CD20pre, by standard Ca²⁺ phosphate precipitation.²⁶ The medium was replaced after 24 hours and finally collected after an additional 24 hours and filtered in the presence of Polybrene. Exponentially growing 38C13 cells (0.5×10^5) were exposed overnight to 1 mL of viral supernatant, washed and expanded in complete RPMI1640. CD20 positive cells were sorted on a FACS sorter (Becton Dickinson, San José, CA, USA) to 98.8% purity after staining with phycoerythrin-labeled anti-CD20 monoclonal antibody (Becton Dickinson). 38C13-CD20⁺ cells were expanded and frozen in aliquots. CD20 expression levels, determined by direct immunofluorescence and FACS analysis were found to be stable for at least 2 months in continuous culture.

In vivo tumor growth

C3H/HeN female mice (4-5 weeks of age) were purchased from Charles River, Calco, Italy. 38C13-CD20⁺ cells

(4×10^5) were inoculated on day 0 in 200 μL of saline by tail vein injection. In parallel groups of mice, 250 μg of rituximab (Roche Italia, Monza, Italy), or saline only, were inoculated intraperitoneally (i.p.) on day 1. In some experiments the same dose of rituximab was given on day 3, 5, 7, 10 or 15. In experiments to test the role of NK cells and neutrophils, groups of mice were inoculated i.p. every 4 days with both the anti-NK cell antibody TM-β1, recognizing the murine interleukin-2 receptor β chain, and anti-GR1 antibody RB6-8C5, specific for murine neutrophils, starting 2 days before tumor cell inoculation, as described elsewhere.²² In order to test the role of complement, mice were treated with 25 U of cobra venom factor (Quidel Corporation, San Diego, CA, USA) or saline only on days 0, 2 and 5 after tumor cell injection.²³ Clodronate for the preparation of clodronate liposomes was a kind gift from Roche Diagnostics (Mannheim, Germany). Clodronate liposomes were prepared as described previously.²⁷ In experiments to test the role of macrophages, groups of mice were treated i.p. with three injections of 150 μL, 60 μL and 60 μL of clodronate-liposomes or phosphate-buffered saline (PBS)-liposomes on day 0, 7, 14, respectively, after tumor inoculum.²⁸ This procedure has been shown to deplete phagocytic macrophages from spleen, liver and lymph nodes.²⁹⁻³² All procedures with animals were carried out in conformity with the institutional guidelines that comply with national and international laws and policies. In some experiments, athymic mice (CD-1 nu/nuBR, Charles river) were inoculated subcutaneously with the standard dose of 4×10^5 38C13-CD20⁺ cells. Rituximab (250 μg) was then given i.p. one day after the tumor and tumor size was measured three times weekly.

Immunohistochemistry of tumor

Lymph nodes from rituximab-treated and untreated mice excised 20 days following tumor cell inoculation were frozen in liquid nitrogen. Seven-micron sections were cut, fixed in 100% acetone and mounted on poly-L-lysine coated slides. The slides were pretreated with citrate buffer in a microwave for 10 minutes and then immunostained using the DAKO ARK Kit (Dako Corporation, Glostrup, Denmark). The primary antibody was the anti-CD20 7D1 clone (Novocastra, Newcastle, UK), used at a 1:25 dilution.²² The slides were developed with 3,3'-diaminobenzidine, counterstained with Mayer hematoxylin, dehydrated through graded alcohol series, clarified in xylene and mounted in EUKIT Balsam (Bioptica, Milan, Italy).

PCR analysis

We collected whole lymph nodes, spleens and bone marrow cells from pairs of rituximab-treated and untreated mice at days 5, 10, 15 and 20 following tumor inoculation. The tissues were homogenized and genomic DNA was immediately purified according to standard SDS/proteinase K extraction procedures. Genomic DNA (500 ng) was amplified by PCR as previously described.²² Briefly, 30 cycles of amplification (95°C- 64°C- 72°C) were carried out using CD20 forward (5'-AATTCAGTAAATGGGACTTTCCCG-3') and reverse primers (5'-ACTATGTGATGTTGGGTCTGGAG-3'). PCR products were separated on a 0.8% agarose gel, denatured in 0.4M NaOH, 0.6 M NaCl followed by treatment in 0.25M Tris-HCl pH 7.5, 1.5

M NaCl, and blotted onto a Genescreen membrane (NEN Life Science Products, Boston, MA, USA). A 0.9 kb fragment carrying the entire human CD20 cDNA sequence was labeled with ^{32}P using the Megaprime DNA Labeling kit (Amersham Biosciences, Little Chalfont, England) and used for hybridization following standard procedures. Membranes were washed in $0.5\times\text{SSC}$ and exposed to X-ray films (XAR5, Kodak). To control the quality and quantity of DNA, the endogenous gene *ptx3* was amplified from the same DNA preparations, as described previously.²² Briefly DNA was amplified with the forward 5'-AGCAATGCACCTCCCTGCGAT-3' and reverse 5'-TCCTCGGTGGGATGAAGTCCA-3' *ptx3* primers for 28 cycles. PCR products were run in an ethidium bromide/1.5% agarose gel.

38C13-CD20⁺ cell growth in vitro

Exponentially growing 38C13-CD20⁺ cells were plated at 5×10^3 /well in 96-well plates in complete RPMI1640 and in the presence or absence of 10 $\mu\text{g}/\text{mL}$ rituximab. After 24, 48 or 72 hours, 1/10 volume alamar blue solution (Biosource, Camarillo, CA, USA) was added in triplicate wells. Incubation was carried out for a further 16 hours and the plates were read in a fluorescence plate reader (Cytofluor 2300, Millipore, Bedford, MA, USA) with excitation at 530 nm and emission at 590 nm.³³ Medium without cells was used to determine background fluorescence, which was subtracted from the values obtained in the presence of cells.

Relocalization of CD20 into detergent insoluble rafts

CD20 was relocalized into lipid rafts essentially as described previously.³⁴ Briefly 2×10^6 38C13-CD20⁺ cells were incubated for 15 minutes at 37°C in the presence or absence of 10 $\mu\text{g}/\text{mL}$ rituximab. Cells were then pelleted and lysed in 20 mM TrisHcCl pH 7.5, 1% Triton X-100, 5 mM EDTA and protease inhibitors for 15 minutes at 4°C. The lysate was centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant was diluted 1:1 in 2 \times concentrated SDS loading buffer. The pellet was washed in lysis buffer and resuspended in SDS loading buffer by 2 cycles of boiling for 5 minutes, vortexing and freezing. Samples were run in a 12% SDS polyacrylamide gel under reducing conditions and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Homogeneous transfer of proteins in all lanes was verified by Ponceau red staining. Blots were blocked in PBS with 5% low fat milk and incubated with 1 $\mu\text{g}/\text{mL}$ goat anti-human CD20 polyclonal antibody (Santa Cruz Biotechnology, CA, USA) and anti-goat-peroxidase conjugated antibody diluted 1:500 (Santa Cruz). Blots were developed using an electrochemiluminescence kit according to the manufacturer's instructions (Amersham).

CDC assay in vitro

Exponentially growing 38C13-CD20⁺ cells were plated at 1×10^6 /mL in 96-well plates in complete RPMI1640 and in the presence or absence of 10 $\mu\text{g}/\text{mL}$ rituximab or the mouse IgG2a anti-CD20 monoclonal antibody 1F5. Rat serum was added at a final concentration of 5-20% and plates were incubated at 37°C for 3 hours. Lysis was measured by staining with propidium iodide and flow cytometry analysis, according to standard procedures.¹⁷

Results

Establishment and characterization of the CD20⁺ B lymphoma model

In order to study the mechanism of action of rituximab *in vivo* in a model most closely mimicking human B-NHL, we used the murine mature B-cell lymphoma cell line 38C13, which metastasizes into lymph nodes.^{35,36} We chose the intravenous route to allow rapid localization of neoplastic cells into hematopoietic organs, including lymph nodes. In order to test the activity of rituximab, which recognizes human, but not mouse CD20,³⁷ 38C13 cells were transduced with a lentiviral vector containing human-CD20 cDNA under the control of a EF1 α promoter.^{25,26} Ninety percent of transduced 38C13 cells expressed CD20 one week after infection (*data not shown*). These were stained with phycoerythrin-labeled anti-CD20 antibody and FACS sorted. Selected cells were expanded and stably expressed the CD20 surface antigen on 100% of the cells, even after 2 months in continuous culture (Figure 1A).

Mean fluorescence intensity for CD20 on the 38C13-CD20⁺ cell line was similar to that observed in human primary follicular lymphoma cells: the mean fluorescence intensity with phycoerythrin-labeled anti-CD20 antibody was 800-850 for 38C13-CD20⁺, compared to a mean of 1220 for ten follicular lymphoma samples tested (*data not shown*).

We next investigated the *in vivo* growth characteristics of the wild type and CD20-transduced 38C13 cell lines after injection of 4×10^5 cells i.v. in syngeneic animals. Both cell lines led to death of 100% of the animals within a median of 21 days with no difference in terms of animal survival and tumor localization (*data not shown*). In both cases, the cells were observed to home to the lymph nodes, as shown by macroscopic examinations of animals at necropsy (Figure 1D and *data not shown*). Immunohistochemistry analysis of lymph nodes obtained 20 days after inoculation of animals with 38C13-CD20⁺ cells showed that lymphoma cells expressing the CD20 antigen had indeed expanded in the lymph nodes of these animals (Figure 1C).

The therapeutic activity of rituximab was then tested: 4×10^5 38C13-CD20⁺ cells were inoculated i.v. on day 0 and a single dose of 250 μg rituximab injected i.p. on day 1. Groups of ten animals were analyzed. As shown in Figure 1B, control animals died a median of 23 days after tumor inoculation, whereas rituximab treatment led to long-term survival of 100% of the animals. This result was highly reproducible. Animals were kept alive for at least three months after tumor cell inoculation and did not show any sign of tumor at necropsy. In contrast, a control IgG1 antibody (anti-CD25 antibody daclizumab) had no therapeutic activity, as expected (*data not shown*). The lymph nodes of tumor-inoculated animals treated with rituximab appeared macroscopically normal (Figure 1D) and did not contain detectable human CD20⁺ cells as determined by FACS analysis (*data not shown*) or by PCR analysis using human CD20 cDNA specific primers (Figure 1D).²²

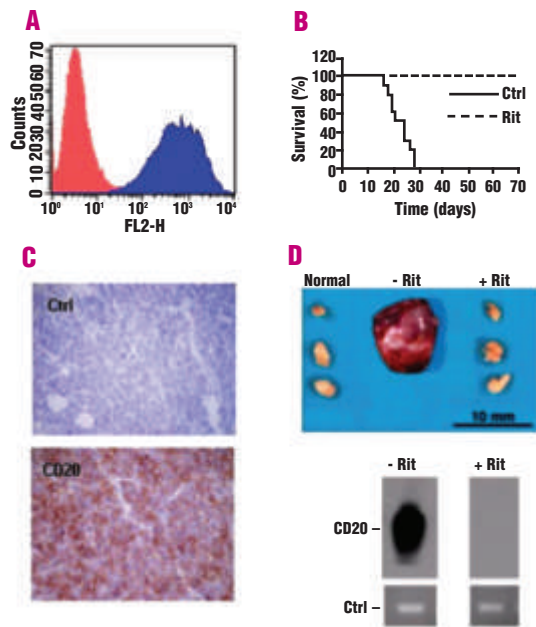


Figure 1. Establishment of the 38C13-CD20⁺ B lymphoma model. (A) Wild type 38C13 cells were infected with a lentiviral vector carrying the complete human CD20 cDNA. The FACS histogram of CD20⁺ selected and expanded cells is shown after staining with phycoerythrin-labeled CD20 antibody (blue profile) or control antibody (pink profile). (B) Survival curves of ten C3H/HeN mice inoculated i.v. with 4×10^3 38C13-CD20⁺ cells and then given 250 µg rituximab i.p. one day after. (C) Lymph nodes from animals which received 38C13-CD20⁺ cells but no rituximab treatment were excised at day 20 and analyzed by immunohistochemistry with an anti-human CD20 antibody. (D) Lymph nodes from control mice or from 38C13-CD20⁺ injected animals treated or not with rituximab on day 1 were collected at day 20 and photographed. DNA was then extracted and analyzed for the presence of the human CD20 cDNA by PCR and Southern blotting with a human cDNA probe.²² The ethidium bromide stained gel of the amplified mouse gene (ptx3) is shown as a control in the lower panel.

Rituximab does not have a direct effect on 38C13-CD20⁺ growth *in vitro* but induces relocalization of human CD20 into lipid rafts and complement-mediated lysis

In order to determine the mechanism of action of rituximab, we first investigated whether rituximab had any direct effect on the growth and viability of 38C13-CD20⁺ cells *in vitro*. 38C13-CD20⁺ cells were plated in the presence or absence of rituximab and cell growth was measured at different times using the alamar blue vital dye. As shown in Figure 2A, rituximab did not alter the growth curve of the cells. These results were confirmed by a manual count of the cells (*data not shown*). We conclude that rituximab does not inhibit 38C13-CD20⁺ cell proliferation or induce apoptosis.

We next determined whether rituximab activates rodent complement *in vitro*. Mouse serum is known to be poorly lytic *in vitro*, so rat serum was used. 38C13-CD20⁺ cells were incubated in the presence or absence of 10 µg/mL rituximab or the mouse IgG2a anti-CD20 monoclonal antibody 1F5. Rat serum was added at a final concentration of 5–20%. The percentage of lysed cells with respect to control samples incubated with serum alone was measured. The data show that rituximab activates rat

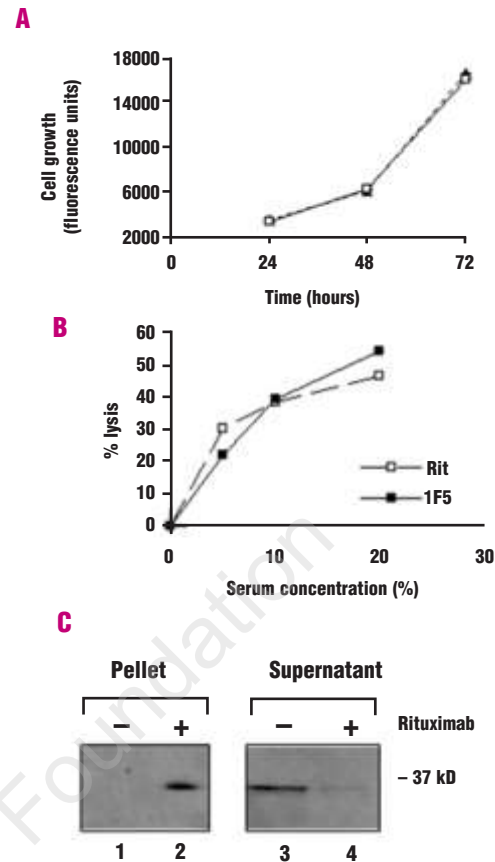


Figure 2. Effects of rituximab *in vitro*. (A) Effect on cell growth: 38C13-CD20⁺ cells were plated in the presence or absence of 10 µg/mL rituximab. After 24, 28 and 72 hours, the number of live cells was evaluated with the vital dye alamar blue and measurement of fluorescence at 590 nm. The results are the mean fluorescence units and standard deviations of triplicate wells in the presence (open squares) or absence (closed triangles) of rituximab. (B) Activation of complement: 38C13-CD20⁺ cells were plated in the presence of increasing concentrations of rat serum and 10 µg/mL of either rituximab (open squares) or mouse anti-CD20 monoclonal antibody 1F5 (closed squares). Percentage cell lysis with respect to controls in the presence of rat serum only was determined after 3 hours by FACS analysis of propidium iodide stained cells. (C) Relocalization of human CD20 into detergent insoluble lipid rafts: 38C13-CD20⁺ cells were incubated in the presence (lanes 2,4) or absence (lanes 1,3) of 10 µg/mL rituximab for 15 minutes at 37 °C. Cells were lysed in 1% Triton-X100. Detergent-insoluble pellets (lanes 1-2) and solubilized cellular components (supernatant, lanes 3-4) were separated in a 12% SDS-PAGE gel, and CD20 analyzed by western blotting using an anti-human CD20 antibody. The results are representative of two independent experiments.

complement to a similar level as the murine anti-CD20 antibody 1F5, leading to lysis ranging from 22% to 54% at different serum concentrations (Figure 2B).

The capacity of different anti-human CD20 antibodies such as rituximab to activate complement has been shown to correlate with relocalization of the target antigen into detergent insoluble lipid rafts.³⁸ We therefore investigated whether rituximab could redistribute human CD20 into lipid rafts also when expressed in a mouse B-cell background. Incubation of 38C13-CD20⁺ cells with rituximab led to rapid redistribution of CD20 into lipid rafts, as shown by an increase in CD20 protein present in

the detergent-insoluble pellet and a decrease in the soluble fraction (Figure 2C). Similar results were obtained using a human B lymphoma cell line (DHL4) whereas the wild type 38C13 cell line showed no detectable band, as expected (*data not shown*). These data show that the transduced human CD20 protein in a mouse B-cell background shows the same physical properties as endogenous CD20 in human B cells in response to rituximab.

Role of immune cells and complement in the therapeutic activity of rituximab

We next investigated the mechanism of action of rituximab *in vivo*. We had previously that depletion of NK cells, polymorphonuclear cells (PMN) or both together with specific antibodies did not affect the therapeutic activity of rituximab in the metastatic EL4-CD20⁺ T lymphoma model.²² We therefore investigated whether these cells played a role in the mechanism of action of rituximab in the 38C13-CD20⁺ B lymphoma model. We first verified that the anti-NK (IL-2R β) and anti-PMN (GR1) antibodies intended to be used for NK and PMN depletion did not bind to the 38C13-CD20⁺ cell line by standard immunofluorescence analysis (*data not shown*). The effect of cell depletion *in vivo* was then analyzed. Groups of mice were treated every 4 days with both anti-NK and anti-PMN antibodies starting 2 days before tumor inoculation, as described previously.^{22,39,40} Rituximab was then administered at the standard dose one day after the tumor inoculation. As shown in Figure 3A, treatment with the two antibodies in the absence of rituximab did not significantly affect tumor growth, the median survival being 21 days without antibodies and 24 days with the anti-NK/PMN treatment ($p=0.5$). In the presence of rituximab, 100% of both groups of animals showed long-term survival (Figure 3A). Thus rituximab retained full efficacy even in NK and PMN depleted mice.

Macrophages express Fc γ Rs and may be involved in antibody-mediated tumor cell destruction. In order to investigate the role of macrophages in the therapeutic activity of rituximab, these cells were depleted by weekly clodronate-liposome *i.p.* treatment, starting on day 0. Mice then received the standard dose of 38C13-CD20⁺ cells on day 0 and rituximab on day 1. As shown in Figure 3B, clodronate-liposomes did not alter tumor growth rate or localization in the absence of rituximab (median survival 22 days with clodronate compared to 24 days without) ($p=0.3$). More importantly, rituximab remained fully active in mice groups treated with either PBS-liposomes or clodronate-liposomes, with long-term survival in 100% of both groups (Figure 3B). In order to determine the role of complement *in vivo* in this B lymphoma model, complement was depleted using CVF, since C1q knock-out animals were not available for this strain of mice. Mice were inoculated with the standard dose of 4×10^3 38C13-CD20⁺ cells and different groups of animals were treated with 25U of CVF or control saline on days 0, 2 and 4 after tumor injection. Rituximab was administered with the standard schedule on day 1. As shown in Figure 3C, CVF treatment alone did not alter the survival curve of tumor-inoculated mice. The median survival of CVF-treated mice was 22 days compared to 21 for the controls. The effect of CVF on rituximab activity was then evaluated. CVF treatment

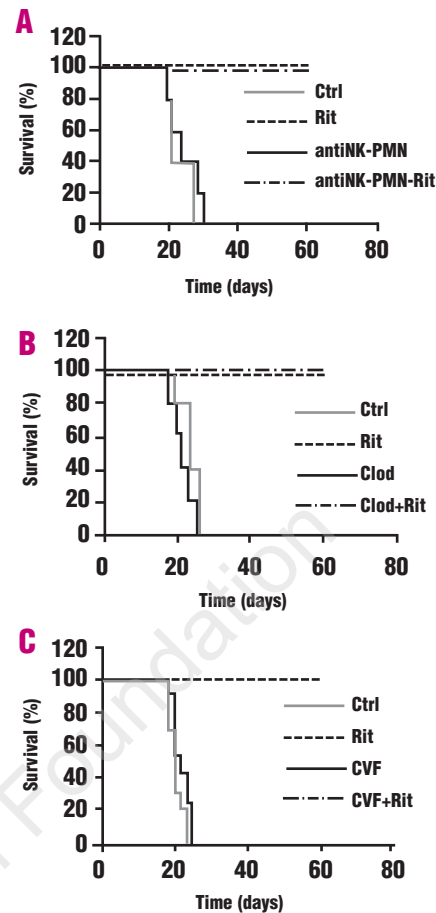


Figure 3. Complement, but not immune cells, is required for the therapeutic activity of rituximab. 38C13-CD20⁺ cells (4×10^3) were inoculated *i.v.* in C3H/HeN mice and 250 μ g rituximab was given *i.p.* one day after. Some groups of animals were pretreated with both anti-NK and anti-PMN antibodies (*panel A*) ($n=5$), some with clodronate-liposomes (Clod) (*panel B*) ($n=5$), and some with CVF (*panel C*) ($n=10$). The survival curves obtained with the different treatments, as indicated in each panel, are shown. The results are representative of at least two independent experiments.

completely abolished the therapeutic activity of rituximab. Indeed 100% of mice treated with CVF and rituximab died within 25 days (median survival 23 days), whereas all mice treated with rituximab and saline showed long-term survival (Figure 3C). Examination of sacrificed animals confirmed the presence of enlarged lymph nodes and spleens in rituximab- and CVF-treated animals in contrast to the macroscopically normal organs in mice treated with rituximab only. These results demonstrate that complement, but not immune cells, is required for the therapeutic activity of rituximab in this B lymphoma model.

Kinetics of tumor growth and rituximab treatment

In order to further characterize the 38C13-CD20⁺ tumor model, we analyzed the therapeutic effect of different rituximab schedules. The standard dose of 4×10^3 38C13-CD20⁺ cells was given, and rituximab was inoculated *i.p.* either 3, 5, 7, 10 or 15 days following tumor cell inoculation (groups of 5 mice). As shown in Figure 4A, rituximab

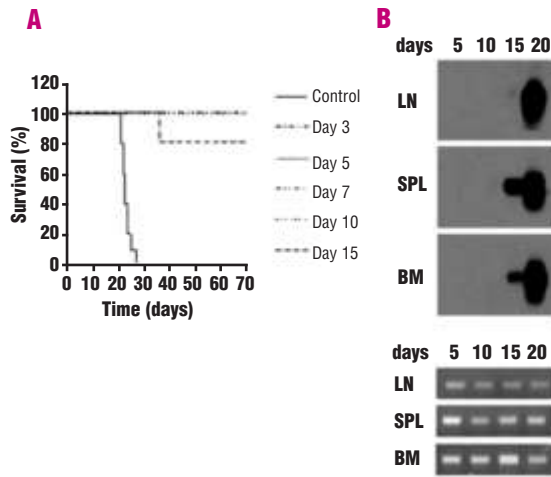


Figure 4. Kinetics of tumor growth and rituximab therapeutic activity. (A) C3H/HeN mice were inoculated i.v. with 4×10^3 38C13-CD20⁺ cells on day 0, followed by 250 μ g rituximab i.p. given on day 3, 5, 7, 10 or 15 (n=5). The survival curves are shown and are representative of two experiments. (B) C3H/HeN mice were inoculated i.v. with 4×10^3 38C14-CD20⁺ cells on day 0. On days 5, 10, 15 and 20, pairs of animals were sacrificed, and lymph nodes (LN), spleen (SPL) and bone marrow (BM) excised for DNA extraction. The presence of CD20⁺ cells was assayed by PCR and hybridization of the PCR products with a human CD20 cDNA probe.²² The same DNA were amplified with an endogenous mouse gene (ptx3) to control their quantity and quality and were run in an ethidium bromide-agarose gel (lower panel). The results are representative of two experiments.

showed full therapeutic activity (100% of animals surviving) when given up to 10 days following tumor cell inoculation. At day 15, rituximab treatment still gave a therapeutic response although it was incomplete (80% long-term survival).

We then studied the kinetics of tumor growth *in vivo*, using PCR analysis and human CD20 cDNA specific primers.²² As shown in Figure 4B, CD20⁺ cells could be detected in the spleen, lymph nodes and bone marrow only around 15 days after tumor inoculation. The cells rapidly expanded thereafter, resulting in a strong PCR signal, corresponding to the grossly enlarged spleen and lymph nodes observed. On the other hand, in animals treated with rituximab a significant CD20 signal was not observed in any organ at any time (Figure 1D and *data not shown*). Amplification of a control murine endogenous gene (ptx3)²² confirmed that all samples carried equivalent amounts of DNA which could be amplified with the same efficiency (Figure 4B, lower panel). Furthermore standard curves performed in parallel with serial dilutions of 38C13-CD20⁺ DNA showed that the limit of detection of CD20⁺ cells was approximately 1 in 10^5 (*data not shown*). Thus these data suggest that inoculated 38C13-CD20⁺ cells migrate to different hematopoietic organs and start expanding to reach detectable levels of 1 in 10^5 cells by about day 15. The cells proliferate rapidly thereafter to form macroscopic tumor masses leading to death of the animals 6-10 days later. Rituximab has full therapeutic activity if administered when cell numbers remain below or around the level of detection but starts to lose effectiveness once the tumor mass begins expanding exponentially.

Since rituximab was still fully effective when injected 10 days after the tumor cells, we also verified the role of com-

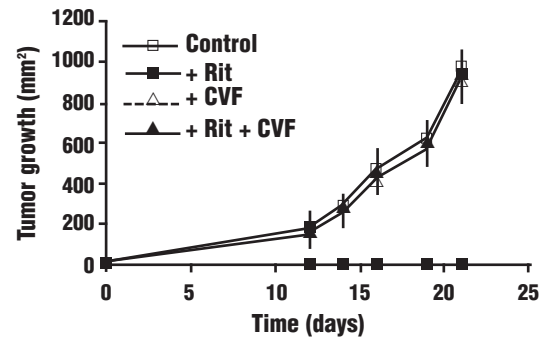


Figure 5. Complement is required for the therapeutic activity of rituximab on subcutaneous 38C13-CD20 tumors. 38C13-CD20⁺ cells (4×10^3) were inoculated subcutaneously into athymic mice (day 0) and rituximab inoculated i.p. one day later (day 1). CVF was given i.p. on days 0, 2, 4 and 9. Tumor growth was measured from day 12 onwards. The curves are the mean and standard deviations of six animals/group.

plement in these conditions. In order to do this animals were inoculated with 38C13-CD20⁺ cells and treated with CVF and rituximab at days 9 and 10 following tumor cell inoculation, respectively. CVF treatment abolished the therapeutic activity of rituximab, as previously observed with the day 1 schedule (*data not shown*).

Complement is required for the therapeutic activity of rituximab in subcutaneous tumors in athymic mice

One possible cause for the differences obtained with this model with respect to some previously published lymphoma xenografts is that in the latter tumors were injected subcutaneously and athymic or SCID mice were used.^{11,12} We have therefore also investigated whether tumor cell localization and/or the mouse strain could lead to different results with regard to the role of complement. The standard dose of 4×10^3 38C13-CD20⁺ cells was injected subcutaneously into athymic mice and the animals were treated with rituximab one day after. Tumor growth was recorded three times a week. As shown in Figure 5, rituximab treatment led to complete eradication of the tumor also in the subcutaneous location, with no sign of tumor growth up to 10 weeks following tumor inoculation. Furthermore, CVF treatment again completely abolished the antibody's therapeutic activity (Figure 5). These results demonstrate that complement is required also in a subcutaneous B-cell tumor and in a different mouse strain.

Discussion

In this report, we have characterized a new *in vivo* model to study the mechanism of action of rituximab, which is centered on the murine B lymphoma cell line 38C13-CD20⁺ that homes to lymph nodes, spleen and bone marrow after i.v. inoculation. Using this model we showed that complement is required for the therapeutic activity of rituximab, whereas immune cells are dispensable. Furthermore we showed the fundamental role of complement even if rituximab treatment was delayed to the tenth day after tumor cell inoculation or if the tumor cells were

injected subcutaneously in athymic mice. The demonstration of the requirement for complement is consistent with our previously described CD20⁺ T lymphoma model, in which wild type or C1q knock-out animals of the C57/BL6 strain were used.²² Furthermore they are consistent with the data of Cragg and Glennie,²³ who showed that complement is required for the therapeutic activity of rituximab in human lymphoma xenografts in immunodeficient mice. Indeed we have confirmed the data of these latter authors in our own xenograft lymphoma model after complement depletion with C56.⁴¹ Thus, put together, these results demonstrate the role of complement in the mechanism of action of rituximab *in vivo* in two syngeneic lymphoma models, one of which homes into lymph nodes as well as in three xenograft models after either subcutaneous or i.v. injection and using four different mouse strains (*this report and work by Di Gaetano²² Cragg²³*). In contrast, Uchida *et al.* recently demonstrated that murine anti-mouse CD20 antibodies do not require complement in order to deplete normal B cells *in vivo*.²⁴ This result is intriguing. A possible explanation for the difference between these results and ours is that Uchida *et al.* investigated depletion of normal B cells, not that of a lymphoma. Perhaps more importantly they used mouse antibodies directed against the mouse CD20 molecule. Also in man, different anti-CD20 antibodies have been shown to have very different properties *in vitro* and *in vivo* according to the epitope that they recognize. Indeed complement activation *in vitro* and the role of complement *in vivo* have been shown to depend dramatically upon the capacity of the therapeutic antibody to translocate CD20 into lipid rafts.^{23,38} Here we have shown that rituximab activates rodent complement after binding to 38C13 CD20⁺ cells. Furthermore we have demonstrated that the human CD20 molecule in the murine 38C13 background behaves in the same way as endogenous human CD20 in that it rapidly relocates into lipid rafts after rituximab binding. Whether the murine anti-mouse CD20 antibodies used by Uchida *et al.* are capable of translocating murine CD20 into lipid rafts and have similar properties as rituximab is not clear at present.²⁴

In contrast to our data showing that immune cells are dispensable for the *in vivo* therapeutic activity of rituximab in the syngeneic mouse models, other authors have pointed to the importance of ADCC rather than complement. They used FcγR knock-out animals¹¹ or depleted NK cells and PMN by antibody treatment as performed here,¹² in the context of xenograft lymphoma models. These authors did not, however, investigate the role of complement, so conclusions cannot be drawn on the role of complement in these settings. Here we showed the lack of requirement for NK cells, PMN as well as macrophages for the therapeutic activity of rituximab, as previously described in the EL4-CD20⁺ model.²² It is possible that the different subsets of leukocytes analyzed, NK, PMN and macrophages, play redundant functions so that depletion of either NK/PMN or macrophages individually may not result in a detectable decrease in therapeutic activity. Another possibility is that residual NK cells, PMN or macrophages remaining after antibody/clodronate treatment, particularly within tissues, participate in tumor cell destruction.^{28,29} Finally, it is also conceivable that the

human Fc portion of rituximab does not interact optimally with mouse Fc receptors. We, however, have evidence that these are unlikely to be valid explanations for our results, since we showed that the same depletion protocol, performed in parallel, was functionally efficacious in another tumor model studied in a mouse background.⁴¹ The characterization of the 38C13-CD20⁺ model presented here may, however, offer another explanation for the differences observed between our syngeneic models and the previously published xenografts. Indeed, the analysis of the kinetics of tumor growth showed that rituximab was active up to 10-15 days after tumor cell inoculation and therefore after the tumor had become established. However the 38C13-CD20⁺ lymphoma is extremely aggressive, with a very rapid proliferation rate *in vitro* and exponential tumor growth measurable from around day 15 onward. Thus we determined that rituximab was still active up to the time at which tumor cells started to reach a PCR-detectable level in different tissues, corresponding to about 1 tumor cell in 10⁵ normal cells. The 38C13-CD20 model may, therefore, represent the effect of rituximab in a situation of minimal residual disease of an aggressive lymphoma. In contrast, xenograft models are slow growing and require a large cellular inoculum to engraft. They may be more representative of larger indolent tumors. Indeed we have evidence in our own bulky lymphoma xenograft model, that not only complement, but also immune cells, are required in this case for tumor control by rituximab.⁴¹ The central and perhaps exclusive role of complement in the control of minimal residual disease is of particular interest in the light of the proposed use and efficacy of rituximab in this kind of clinical context,⁴² such as in patients receiving high dose chemotherapy for disease control and stem cell transplantation and who may, therefore, have low numbers and/or ineffective immune cells to enable ADCC.⁴³

Altogether these considerations suggest that complement is required universally for the therapeutic activity of rituximab in different lymphoma models showing different localizations, and may be sufficient by itself to control minimal residual disease. The importance of complement activation should not, therefore, be undervalued in the design of novel therapeutic monoclonal antibodies, for which modifications to increase their capacity to induce ADCC have been attempted.⁴⁴ Although rapid complement activation *in vivo* is also likely to be the major cause of the commonly observed infusion-related side-effects of rituximab,^{20,21} these potentially severe complications are efficiently counteracted by slower or fractionated drug administration as well as by adequate pre-medication.³

JG and MI designed and supervised the project and found grant support. JG wrote the manuscript. EC, NDG and MC performed the in vivo and in vitro studies. EC, MN and LC performed and evaluated immunohistochemistry experiments. MM generated the 38C13-CD20 cell line. NvR provided reagents. The authors declare that they have no potential conflicts of interest.

This work was supported by the Italian Association for Cancer Research (AIRC), the Italian Ministry for University and Research (FIRB projects RBAU01J2ER and RBAU01H8SX), the Associazione Italiana per la Lotta alla Leucemia (AIL) - Sezione Paolo Belli, and by Roche Italia.

Manuscript received June 7, 2005. Accepted November 25, 2005.

References

- Byrd JC, Stilgenbauer S, Flinn IW. Chronic lymphocytic leukemia. *Hematology (Am Soc Hematol Educ Program)* 2004; p. 163-83.
- Thomas DA, O'Brien S, Bueso-Ramos C, Faderl S, Keating MJ, Giles FJ, et al. Rituximab in relapsed or refractory hairy cell leukemia. *Blood* 2003;102:3906-11.
- Boye J, Elter T, Engert A. An overview of the current clinical use of the anti-CD20 monoclonal antibody rituximab. *Ann Oncol* 2003;14:520-35.
- Lin TS, Lucas MS, Byrd JC. Rituximab in B-cell chronic lymphocytic leukemia. *Semin Oncol* 2003;30:483-92.
- Feugier P, Van Hoof A, Sebban C, Solal-Celigny P, Bouabdallah R, Ferme C, et al. Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. *J Clin Oncol* 2005;23:4117-26.
- Treon SP, Hansen M, Branagan AR, Verselis S, Emmanouilides C, Kimby E, et al. Polymorphisms in FcγRIIIa (CD16) receptor expression are associated with clinical response to rituximab in Waldenström's macroglobulinemia. *J Clin Oncol* 2005;23:474-81.
- Cooper N, Stasi R, Cunningham-Rundles S, Feuerstein MA, Leonard JP, Amadori S, et al. The efficacy and safety of B-cell depletion with anti-CD20 monoclonal antibody in adults with chronic immune thrombocytopenic purpura. *Br J Haematol* 2004;125:232-9.
- Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med* 2004;350:2572-81.
- Cartron G, Watier H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. *Blood* 2004;104:2635-42.
- Maloney DG, Smith B, Rose A. Rituximab: mechanism of action and resistance. *Semin Oncol* 2002;29 Suppl 2:2-9.
- Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat Med* 2000;6:443-6.
- Hernandez-Ilizaliturri FJ, Jupudy V, Ostberg J, Oflazoglu E, Huberman A, Repasky E, et al. Neutrophils contribute to the biological antitumor activity of rituximab in a non-Hodgkin's lymphoma severe combined immunodeficiency mouse model. *Clin Cancer Res* 2003;9:5866-73.
- Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγIIIa gene. *Blood* 2002;99:754-8.
- Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 2003;21:3940-7.
- Farag SS, Flinn IW, Modali R, Lehman TA, Young D, Byrd JC. FcγRIIIa and FcγRIIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia. *Blood* 2004;103:1472-4.
- Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab *in vitro*: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 2000;95:3900-8.
- Golay J, Lazzari M, Facchinetti V, Bernasconi S, Borleri G, Barbui T, et al. CD20 levels determine the *in vitro* susceptibility to rituximab and complement of B-cell chronic lymphocytic leukaemia: further regulation by CD55 and CD59. *Blood* 2001;98:3383-9.
- Kennedy AD, Solga MD, Schuman TA, Chi AW, Lindorfer MA, Sutherland WM, et al. An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20+ cells by rituximab. *Blood* 2003;101:1071-9.
- Manches O, Lui G, Chaperot L, Gressin R, Molens JP, Jacob MC, et al. *In vitro* mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 2003;101:949-54.
- Kennedy AD, Beum PV, Solga MD, DiLillo DJ, Lindorfer MA, Hess CE, et al. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J Immunol* 2004;172:3280-8.
- van der Kolk LE, Grillo-Lopez AJ, Baars JW, Hack CE, van Oers MH. Complement activation plays a key role in the side-effects of rituximab treatment. *Br J Haematol* 2001;115:807-11.
- Di Gaetano N, Cittera E, Nota R, Vecchi A, Grieco V, Scanziani E, et al. Complement activation determines the therapeutic activity of rituximab *in vivo*. *J Immunol* 2003;171:1581-7.
- Cragg MS, Glennie M. Antibody specificity controls *in vivo* effector mechanisms of anti-CD20 reagents. *Blood* 2003;103:1238-43.
- Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, et al. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 2004;199:1659-69.
- Serafini M, Bonamino M, Golay J, Introna M. Elongation factor 1 (EF1α) promoter in a lentiviral backbone improves expression of the CD20 suicide gene in primary T lymphocytes allowing efficient rituximab-mediated lysis. *Haematologica* 2004;89:86-95.
- Manganini M, Serafini M, Bambacioni F, Casati C, Erba E, Follenzi A, et al. A human immunodeficiency virus type 1 pol gene-derived sequence (cPPT/CTS) increases the efficiency of transduction of human nondividing monocytes and T lymphocytes by lentiviral vectors. *Hum Gene Ther* 2002;13:1793-807.
- Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 1994;174:83-93.
- Fraser CC, Chen BP, Webb S, van Rooijen N, Kraal G. Circulation of human hematopoietic cells in severe combined immunodeficient mice after Cl2MDP-liposome-mediated macrophage depletion. *Blood* 1995;86:183-92.
- van Rooijen N, Sanders A. Elimination, blocking, and activation of macrophages: three of a kind? *J Leukoc Biol* 1997;62:702-9.
- Biewenga J, van der Ende MB, Krist LF, Borst A, Ghuftron M, van Rooijen N. Macrophage depletion in the rat after intraperitoneal administration of liposome-encapsulated clodronate: depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by administration of Freund's adjuvant. *Cell Tissue Res* 1995;280:189-96.
- Jun HS, Yoon CS, Zbytniuk L, van Rooijen N, Yoon JW. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Exp Med* 1999;189:347-58.
- van Rooijen N, van Kesteren-Hendrikx E. "In vivo" depletion of macrophages by liposome-mediated "suicide". *Methods Enzymol* 2003;373:3-16.
- Golay J, Gramigna R, Facchinetti V, Capello D, Gaidano G, Introna M. Acquired immunodeficiency syndrome-associated lymphomas are efficiently lysed through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity by rituximab. *Br J Haematol* 2002;119:923-9.
- Deans JP, Robbins SM, Polyak MJ, Savage JA. Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. *J Biol Chem* 1998;273:344-348.
- Maloney DG, Kaminski MS, Burowski D, Haimovich J, Levy R. Monoclonal anti-idiotypic antibodies against the murine B cell lymphoma 38C13: characterization and use as probes for the biology of the tumor *in vivo* and *in vitro*. *Hybridoma* 1985;4:191-209.
- Aviram R, Raz N, Kukulansky T, Hollander N. Expression of L-selectin and efficient binding to high endothelial venules do not modulate the dissemination potential of murine B-cell lymphoma. *Cancer Immunol Immunother* 2001;50:61-8.
- Polyak MJ, Deans JP. Alanine-170 and proline-172 are critical determinants for extracellular CD20 epitopes; heterogeneity in the fine specificity of CD20 monoclonal antibodies is defined by additional requirements imposed by both amino acid sequence and quaternary structure. *Blood* 2002;99:3256-62.
- Cragg MS, Morgan SM, Chan HT, Morgan BP, Filatov AV, Johnson PW, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. *Blood* 2003;101:1045-52.
- Pruitt JFM, Verzaal P, van Os R, de Kuijff EFM, van Schie MLJ, Mantovani A, et al. Neutrophils are indispensable for hematopoietic stem cell mobilization induced by interleukin-8 in mice. *Proc Natl Acad Sci USA* 2002;99:6228-33.
- Tanaka T, Kitamura F, Nagasaka Y, Kuida K, Suwa H, Miyasaka M. 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* 1993;178:1103-7.
- Golay J, Cittera E, Di Gaetano N, Nebuloni M, Vago L, Vecchi A, et al. The mechanism of action of rituximab *in vivo* depends on the tumor model. *Haematologica* 2005;90 (S2):325a [abstract].
- Brugger W, Hirsch J, Grunebach F, Repp R, Brossart P, Vogel W, et al. Rituximab consolidation after high-dose chemotherapy and autologous blood stem cell transplantation in follicular and mantle cell lymphoma: a prospective, multicenter phase II study. *Ann Oncol* 2004;15:1691-8.
- Khoury IF, Saliba RM, Hosing C, Okoroji GJ, Acholonu S, Anderlini P, et al. Concurrent administration of high-dose rituximab before and after autologous stem-cell transplantation for relapsed aggressive B-cell non-Hodgkin's lymphomas. *J Clin Oncol* 2005;23:2240-7.
- Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. *J Biol Chem* 2002;277:26733-40.